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VOLUME 4 JANUARY, 1931 NUMBER 1

THE NATURE OF THE RESINS IN JACK PINE (*PINUS BANKSIANA*)[1]

BY HAROLD HIBBERT[2] AND JOHN BERNARD PHILLIPS[3]

Abstract

Green and seasoned jack pine were found to have an average crude resin content of 4.58% and an ether-soluble of 3.52%. The value of "total acids" present in the resin was the same in each case, but the proportion of "fatty" acids was greater in green wood, while the unsaponifiable matter was considerably less. The seasoned wood contained a lower percentage of fats than the green wood and a correspondingly higher percentage of resin acids. The amount of unsaponifiable, polymerized terpenic substances was also higher. There was a marked decrease in the amounts of phytosterol and "resene" in the seasoned wood.

The isolated resin acids obtained by distillation, or esterification, contained a high percentage of abietic acid formed by transformation of the original acids. The percentage of natural (pimaric) acids was, however, quite high in the resin acids isolated by petroleum ether and recrystallized only twice.

A higher percentage of crystalline acids was found in the resin acids from green wood than from seasoned, due presumably to change into amorphous products during storage.

The fatty constituents were chiefly members of the unsaturated series, present both as free acids, glycerides, or other esters. The seasoned wood contained much less linolic acid in the glycerides than green wood. Oleic acid was present in about the same proportion in both cases; linolenic was present in only very small amount. The free fatty acids had practically the same percentage composition in both green and seasoned wood.

The amount of the total unsaturated fatty acids was higher in the glycerides and free acids from green wood than from seasoned wood. The latter was found to contain a high percentage (30.2%) of oxidized acidic material in the fatty glycerides, indicating that extensive polymerization, or decomposition of some kind, had taken place in the fats present in the green wood during the time of seasoning.

The quantities of essential oil obtained from the resins of each kind of wood were very small, amounting to 1.5 to 4.3% of the total crude resins. The products showed no difference in properties, or variation in amount, with time of storage of the wood. Owing to the small amount available for investigation, only α-pinene could be identified, although other terpenes may possibly be present.

The percentages of phytosterol and resene were extremely small, and were appreciably less in the seasoned than in the green wood. The amount of polymerized terpenic material found in the unsaponifiable matter was much higher in the case of the seasoned wood. This was probably due to extensive polymerization of the essential oil having taken place during the storage of the wood.

[1] *Manuscript received November 11, 1930.*

Contribution from the Industrial and Cellulose Laboratories, McGill University, Montreal, Canada. Constructed from a thesis presented to the Graduate Faculty of McGill University in May 1930 by J. B. P. in partial fulfilment of the requirements for the Ph.D. degree.

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Introduction

The resins of jack pine (*Pinus banksiana*) are of peculiar interest both scientifically and industrially, because of the fact that the inner secretions of the conifers, often referred to as "physiological resins", have been investigated thoroughly only in very rare instances, and also on account of the increasing importance of this wood in the pulp and paper industry. The oleoresins of certain species of the genus *Pinus*, which are of great importance in the Naval Stores industry, have been the subject of considerable investigation for many years. While a large supply of working material is readily available in the case of the oleoresins, the inner secretions are present in the wood usually in very small proportions, so that isolation and identification of the individual constituents of the complex mixture is a difficult matter.

Some important facts concerning many of the conifers of North America were obtained by Schorger (37) in his investigations of the oils and oleoresins from various parts of the trees. The long-leaf pine (*Pinus palustris*), a species very important in the American turpentine and rosin industry, was investigated by Bates (9) with regard to the chemical utilization of the resins and other products from the waste wood.

Although jack pine is widely and abundantly distributed in Canada and certain parts of the United States, no information has been hitherto available regarding the actual composition of the resins of this species. The amounts of resinous matter removed by ether and alcohol from certain specimens of jack pine have been determined (8).

The resin acids, fats, essential oils and unsaponifiable matter constitute the greater part of the crude, resinous material ordinarily extracted from coniferous woods.

Resin Acids

The resin acids of the conifers have been carefully investigated in certain cases, especially those of the oleoresinous species (15, 29, 34, 40, pp. 623-725). These resin acids have been classified by Klason and Köhler (26) as follows.

(1) "Natural acids", in the original state, consisting of:

(*a*) Sapinic acids, which are extremely unstable, and have never been isolated in a pure state; their structure is unknown. The sodium salts of the sapinic acids are very soluble compared with those of the pimaric acids.

(*b*) Pimaric acids; the levo- form is unstable, but the dextro- acid is quite stable, and can be distilled unchanged under reduced pressure. The structure of these acids has not been established definitely.

(2) "Colophenic acids", which are transformation products of the natural acids; this class includes the different forms of abietic acid. Abietic acid is the best known of the resin acids, and its structure has been established (34).

The classification given above has been extended in some respects by Aschan (6).

The colophenic acids are considered to be, in general, isomeric with the natural acids, but have different properties. The pimaric acids are distin-

guished by the fact that their ammonium salts are crystalline, while those of the colophenic acids are gelatinous (15, 26). The colophenic acids (abietic) have the property of forming crystalline acid sodium salts, while the natural acids do not; this property has been used in the quantitative estimation of abietic acid (16).

The natural resin acids in general are very susceptible to the action of light, heat, air, and chemical reagents, and are extremely difficult to isolate in unchanged condition. They are readily transformed to isomeric forms which are often amorphous.

Fats and Fatty Acids

The inner secretions of conifers generally contain appreciable quantities of fats and fatty acids. These have never been investigated thoroughly, except for certain work which has been carried out in Europe on by-products (pine oil, "tallöl", etc.) of the soda and sulphate pulping processes. The unsaturated acids of the oleic series have been reported as present in these products (10, 14, 35) as well as certain other fatty acids which are probably formed from the above during the chemical pulping process (13, 33). Some of the latter substances have not been fully identified (22).

Some investigations have been carried out on the variation of resin and fat content with time of seasoning of the wood, and some speculations made concerning the changes which occur in these substances with time (38), but no definite conclusions can be drawn from this work. The results are confusing on account of the fact that a wide variety of experimental conditions have been employed by different workers.

Essential Oils

Volatile essential oils are present in the woody tissues of conifers, but usually in such small amounts as to make difficult any attempt at a thorough investigation of the constituents. Pine and spruce chips yield only traces of volatile turpentine oil, even when distilled with alkali under high pressure. The essential oils of the most important oleoresins have been very thoroughly investigated, but these are not necessarily of the same chemical composition as the oils secreted in the inner woody tissues. Pinene, dipentene, and sylvestrene have been reported (5, pp. 21, 1447) as being present in the wood-resins of certain conifers; pinene is also generally the principal constituent of the oils of the oleoresins.

Unsaponifiable Matter

Resins contain certain other materials, classed as unsaponifiable matter. The composition and amount of this portion varies with the kind of resin. The nature of the substances in this class present in the resins of the woody tissues has never been elucidated. Sterols have been isolated in some instances from the by-products of wood-pulping processes (10, 14, 35). The resenes or unsaponifiable matter of various resins have been isolated (24, 40, pp. 1079-82), but the chemical composition has never been established. The percentage of unsaponifiable matter in most resins is small.

Extraction

The only efficient method of obtaining these internal secretions from the woody tissues is by extracting with organic solvents. Various extraneous components of the wood are also soluble in these solvents, and for this reason, the extracted crude material is a very complex mixture consisting, in general, of (a) resin acids; (b) fatty acids, esters and glycerides; (c) essential oils; (d) unsaponifiable matter; (e) tannins, dye-stuffs, etc.; (f) lignin; (g) sugars and similar substances.

Methods of Separation

There is no systematic general procedure known for separating such a variety of natural substances from each other and subsequently identifying the individual constituents. Two methods have been used previously in the examination of resins for effecting an approximate separation into groups of related substances, and each of these methods was examined carefully in the present investigation.

(1) One of these is a general method for the examination of rosin, mixtures of rosin and fats, crude resins, etc., and has been adapted by Sieber (38, pp. 136-137) to the proximate analysis of conifer resins. This method involves a preliminary hot saponification of the total crude extracted resin product, removal of unsaponifiable matter by petroleum ether extraction, and separation of the acidic products into (a) resin acids and (b) fatty acids.

In the present investigation, the analytical results were found to check satisfactorily when Sieber's procedure was carefully followed. Appreciable losses in water-soluble material were noted, probably due to hydrolysis of the glycerides or decomposition of the lignin, the latter being present in fairly large proportion in the crude resins. The percentage of water-soluble materials was calculated by difference in all cases.

The precipitation of insoluble material at different stages in the separation is a disadvantage of this method. The saponification treatment does not permit of the isolation of glycerides, esters, etc., as such. The small amount of essential oil present is also polymerized by this treatment, so cannot be isolated or identified.

The natural substances of which these resins consist are, in general, quite sensitive to heat, chemical reagents and other influences, and it is probable that they undergo fundamental changes when treated by the above method. It does not seem to be suitable where a subsequent investigation of the individual constituents is intended.

(2) The other method of separation is that employed by Tschirch and coworkers (40, pp. 60-62, 546, 623-725) on oleoresins and rosin from various species of conifers. This method involves a long series of treatments with certain aqueous alkaline solutions, namely, 1% ammonium carbonate, 1% sodium carbonate, 1% potassium hydroxide, and 10% potassium hydroxide, used successively. These extract the acidic substances from the resins, and the acids are recovered from the alkaline solutions. The essential oil can be recovered from the residue by steam-distillation, leaving the unsaponifiable

portion called the resene by Tschirch. Various other means were used by Tschirch in endeavoring to obtain these products in a pure state.

This method of separation is very useful and efficient for estimating the amounts of certain substances present, but not for the identification of the individual constituents as it does not afford a sharp separation of the different types of substances. The isolation of the pure resin acids by this method is difficult, and there is no provision for the isolation of the large amounts of fatty substances present in wood-resins. The procedure of exhaustive extraction with four different alkaline solutions, and separate treatment of each of the extracts later, is a very tedious process, and there is no satisfactory proof that the various acidic products obtained in this manner are pure substances chemically distinct from each other.

The four acidic products obtained by this method in the present investigation from one lot of the resins were examined individually, and found to contain, in each case, considerable amounts of fatty substances, in addition to the resin acids portion (Table IV). These products had to be separated by a chemical process (41). Furthermore, the use of alkaline solutions of 1% strength results in very large volumes of extracts where comparatively large amounts of resins are being treated, while the final treatment with 10% potash apparently saponifies the glycerides and esters to some extent, and may also exert a resinifying effect.

Two methods employed by previous workers for separating the fatty from the resin acids were examined; (a) the original method of Twitchell (41), and (b) the modification of this method proposed by Wolff and Scholze (46). The former involves a lengthy saturation treatment with hydrogen chloride gas, and this probably has an isomerizing effect on the resin acids. The shorter treatment by the Wolff and Scholze method, using a small amount of sulphuric acid, probably has less effect on these acidic products. This procedure is also much more rapid and convenient, and gives consistent results.

(3) The general methods for the investigation of resins that have been outlined above were modified and extended so as to form the basis of a more suitable method for examining the resins of jack pine. A new method for separating the crude resinous material was finally adopted for use, and extended to include the isolation and identification of the individual constituents of the various groups of products.

This method is described in the Experimental Part (Diagram I). It was found to be quite effective for the mixture under examination, and enabled the work of separation to be accomplished in much less time than by Tschirch's method. The complete removal of the lignin and other insoluble material by an initial treatment with dilute potassium hydroxide, reprecipitation by acid, and recovery of the residual resins by ether extraction, offers an efficient means of freeing the resins completely from the insoluble impurities and in the shortest time. The use of 5% sodium carbonate solution for extracting the acidic constituents results in much smaller volumes of extract to be handled than when the three dilute solutions recommended by Tschirch are used, and

the exhaustive extraction is completed in much less time. The esters and glycerides are not affected by this strength of alkali, and no trouble is encountered with emulsions. Precipitation of the sodium carbonate extract by acetic acid prevents the isomerization of the resin acids which is found to occur when strong mineral acids are employed.

Resins

The resins from two lots of jack pine wood were investigated in the present work: (*a*) green jack pine, obtained from a tree felled from 10 days to 16 months prior to use; and (*b*) seasoned jack pine, felled five years previously, the log having been stored outdoors since that time, fully exposed to the weather. The trees in each case were from 125 to 140 years old. The wood was completely identified as jack pine (*Pinus banksiana*) by its microscopic features (32, 36) before being used.

A mixture of alcohol and benzene, 50% each by volume, was chosen as the most suitable solvent for removing the resins from the wood. The crude resinous material remaining after removal of the solvent was separated, according to the scheme outlined in Diagram 1, into groups of substances, as follows, each of which was examined in detail.

(*a*) Soluble in sodium carbonate: resin acids and free fatty acids.

(*b*) Insoluble in sodium carbonate: fats and esters (saponifiable); essential oils (volatile); non-volatile and unsaponifiable matter.

Experimental Part I

Extraction and Separation of Crude Resin Product into Groups of Substances

The wood was prepared for extraction by scraping the bark from the log just before the wood was used. The log was then cut into very thin, small shavings by means of a mechanical planer, and these were kept in closed boxes, stored in a cold room, until they were put into the extractor.

The loss in weight at 105° C. was determined on separate samples, representative of the whole batch of wood being treated, by drying in the air oven at 105°C. for exactly 4 hr. This was done at the same time as the moist wood was being weighed, just before loading it into the extractor. Yields of resins were calculated on the "bone-dry weight" of wood thus determined.

The prepared wood was extracted with a mixture of equal volumes of benzene and 95% ethyl alcohol. The extraction was carried out in a large bronze extractor, operating on the Soxhlet principle, in which several hundred grams of wood could be treated at one time. The same lot of solvent was used on three different lots of wood, and then it was replaced by a new alcohol-benzene mixture, in order to avoid the effects of too prolonged heating of the dissolved resins. The extraction was completed in 8-10 hr. in this apparatus.

The alcohol-benzene extract was evaporated on the water bath under slightly reduced pressure in a current of carbon dioxide gas, the solution being kept shielded from light. The residue was carefully dried to constant weight under

reduced pressure. The product (A) was a very viscous, dark brown mass, having the odor peculiar to resinous pine wood.

Preliminary Separation of the Total Crude Resins

Three methods were tried on various lots of the crude resins.

Method A. Saponification with $N/2$ sodium hydroxide, etc., according to the procedure given by Sieber (38, pp. 136-37).

Method B. The general procedure for separation of conifer resins given by Tschirch (40, pp. 60-62, 546, 623-725).

Method C. This was developed in the present investigation and was carried out as follows:

The crude resin product (A) was extracted by refluxing about six times with a large volume of boiling ether. The ethereal extract (a), containing pure resin material, was decanted each time. The insoluble cake (b), finally left behind, was broken up, washed well with ether and then with water, the ether washings being added to the main ethereal extract (a) above, while the water washings (c) were kept for examination. The cake (b) was then dissolved in 1% aqueous potassium hydroxide, reprecipitated by addition of glacial acetic acid, and the mixture extracted with ether. These ethereal extracts, containing the last traces of the resins, were also added to the main ether solution of the resins (a). This process of reprecipitation, etc., was repeated three times, and the final insoluble residue (b′) consisting of lignin, etc. kept for further examination.

The total ether extract of the resins (a′), consisting of the extracts (a) together with the washings, etc., was filtered, and the clear solution shaken repeatedly with small portions of distilled water until all water-soluble material had been removed. This aqueous extract was added to the water-washings (c) and the total extract (c′) examined later for water-soluble components.

The washed ethereal solution (a′) was then exhaustively extracted with portions of 5% aqueous sodium carbonate, using a mechanical shaker. These aqueous alkaline extracts (d) were united and allowed to stand overnight. Considerable amounts of neutral oily material separated as an upper layer and this portion was siphoned off, and again extracted with ether. This ether extract was united with the main ethereal solution (a′) while the aqueous layer was returned to the main sodium carbonate extract (d).

The combined ether extract was washed with water until neutral, and the ether removed by evaporation, leaving a dark brown, viscous residue. This was steam-distilled to obtain the volatile essential oil. The aqueous layer of the steam distillate was extracted with ether to recover more of the oil, and the ethereal extract dried over calcium chloride and evaporated. The oil was purified by distillation at a very low pressure.

The residue left after steam-distillation was extracted with ether, the aqueous layer (e) being kept for determination of glycerol. The ether was evaporated, and the residue (f) saponified for one hour on the water bath with normal alcoholic potash. Extraction of the mixture with ether removed the unsaponifiable product. The ethereal extract (g) was washed with 10%

aqueous alcohol until neutral, and the washings added to the alkaline portion containing the potassium salts of the acids. The washed ethereal extract (g) was dried over calcium chloride, the ether removed, and the unsaponifiable matter dried to constant weight under reduced pressure.

The combined alkaline portion from the saponification was treated with glacial acetic acid, and the liberated acids taken up with ether, while the aqueous residue was added to (e) above, the combined solution being then employed for the determination of glycerol. The ethereal extract was washed with water, dried over fused sodium sulphate and evaporated. A dark brown, oily residue of acids remained, and this was dried to constant weight under reduced pressure.

The sodium carbonate extract (d), containing only the free acid constituents of the resins, was precipitated by glacial acetic acid. The mixture (h) of fatty and resin acids thus obtained was removed by ether extraction, employing repeated mechanical shaking. A dark red aqueous solution remained, from which nothing further could be removed by ether. The ether solution of the total acids (h) was washed with water, dried over fused sodium sulphate, and evaporated, leaving the acids as a dark brown oily mass. These were then separated into fatty and resin acids by the preferential esterification method already mentioned (46), glacial acetic acid being used instead of mineral acids for precipitating the resin acids.

TABLE I

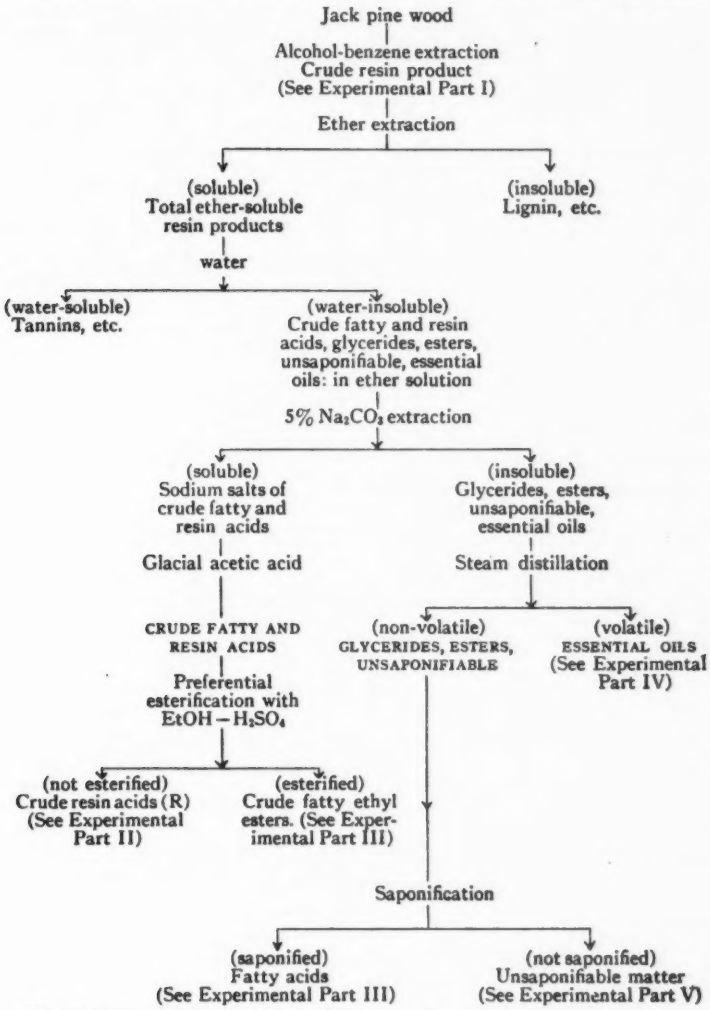
EXTRACTION OF RESINS FROM JACK PINE

Extraction No.	Kind of wood	Time since tree felled, in days	Wood, loss at 105°C. (4 hr.) %	Weight of wood (bone dry) in gm.	Weight of crude resins in gm.	Crude resins %	Ether-soluble resins %
1	Green jack pine	10	38.9	557	26.5	4.76	3.37
3	"	15	9.1	541	25.4	4.70	3.75
4	"	19	6.8	575	26.0	4.52	3.51
5	"	74	6.9	1107	50.6	4.57	3.12
6	"	80	7.3	1079	56.4	5.22	3.12
8	"	89	7.3	317	17.7	5.58	4.71
9	"	90	19.6	2543	113.3	4.45	3.12
10	"	92	18.7	1218	51.7	4.25	3.95
11	"	94	18.7	766	28.5	3.72	3.26
12	"	16 months	11.3	1720	76.8	4.46	—
13	Seasoned jack pine	5 years	13.2	13,400	569.0	4.21	3.31

The entire process of separation of the total resins, as described above, is outlined briefly in the following diagram.

DIAGRAM I

METHOD C FOR SEPARATION OF THE CRUDE RESIN PRODUCT INTO GROUPS OF SUBSTANCES



The subsequent treatment of each of these groups is outlined in Diagrams II, III, and IV

TABLE II

SUMMARY OF ANALYTICAL RESULTS, BY METHOD A, IN THE INITIAL SEPARATION OF CRUDE PRODUCTS FROM VARIOUS EXTRACTIONS OF GREEN JACK PINE

Total crude resins, etc. from Extraction No.	Saponifiable products weighed as total acids and including lignin, insoluble, etc. mean %	Unsaponifiable matter and essential oil mean %	Water soluble material (by difference) %	Separate analysis of saponifiable products including lignin, etc.* mean %		
				Resin acids	Fatty acids	Lignin
1	78.40	4.83	16.8	54.5	30.2	15.1
2	79.0	4.39	16.6	41.6	32.9	21.7
3	81.3	4.78	13.9	42.1	42.3	7.80
4	78.1	5.45	16.4	45.4	38.4	7.5
11	79.3	5.26	15.4	53.2	23.8	6.2

**See also Table VI.*

TABLE III

SUMMARY OF ANALYTICAL RESULTS BY TSCHIRCH PROCEDURE, METHOD B, IN THE SEPARATION OF THE TOTAL CRUDE RESINOUS PRODUCT FROM VARIOUS EXTRACTIONS OF GREEN JACK PINE

Crude material from Extraction No.	Fraction (a)	Fraction (b)				Fraction (c)	
	Product insoluble in ether, in %	Product removed by 1% ammonium carbonate in %	Product removed by 1% sodium carbonate in %	Product removed by 1% potassium hydroxide in %	Product removed by 10% potassium hydroxide in %	Glycerides, unsaponifiable matter, volatile, essential oil, in %	Total water soluble, by difference, in %
3	13.2	23.5	34.3	18.1	—	7.22	10.9
8*	11.3	12.4	43.5	11.3	3.1	14.10	4.3
10	7.2	15.5	52.6	11.0	—	7.22	6.4

**See also Table IV.*

TABLE IV

INDIVIDUAL ANALYSIS OF FRACTION (B), TABLE III, FOR EXTRACTION NO. 8 ONLY. SEPARATION INTO FATTY AND RESIN ACIDS BY TWITCHELL'S METHOD

Product, acids removed by	Fatty acids %	Resin acids %
1% Ammonium carbonate	40.5	59.5
1% Sodium carbonate	34.1	65.9
1% Potassium hydroxide	53.1	46.8
10% Potassium hydroxide	62.8	37.2
Mean %, from total weights of acids	38.2	61.8

TABLE V

SUMMARY OF ANALYTICAL RESULTS, BY METHOD C, IN THE INITIAL SEPARATION OF CRUDE PRODUCTS FROM VARIOUS EXTRACTIONS OF JACK PINE WOOD

Extraction No.	Lignin, etc. insoluble in ether %	Total free acids from 5% Na_2CO_3 extraction %	Glycerides calculated as triolein, approximate %	Unsaponifiable matter only %	Essential oil %	Water-soluble material by difference %	Analysis of total free acids only	
							Resin acids %	Fatty acids %
Green wood								
9x	9.2	66.0	7.6	4.5	1.5	8.3	81.6	18.3
Seasoned wood								
13 (a)	6.9	62.4	4.7	7.71	1.4	13.2	87.5	12.5
13 (b)	8.1	62.6	6.4	7.96	2.1	13.8	86.1	13.9
Mean %	7.5	62.5	5.6	7.84	1.7	13.5	86.6	13.2

NOTE: (*a*) *The crude resin products from Extractions 5, 6, 7 and 9, (Table I) from green wood were combined for analysis, and the results reported above as Extraction 9x.*
(*b*) *Analysis of the crude resins from seasoned wood, (Extraction 13, Table I) was carried out on two separate lots, No. 13 (a) and 13 (b) above. The mean percentages of constituents are also given above.*

TABLE VI

GENERAL SUMMARY OF ANALYSES OF CRUDE PRODUCTS

Crude product (See Table I) Extraction No.	Kind of wood	Water-soluble (tannins, carbohydrates, etc.) %	Ether-insoluble (lignin, etc.) %	Resin acids %	Fatty acids %	Glycerides and esters, %	Volatile essential oil (av.) %	Polymerized terpenic substances etc. (av.) %	Phytosterols (av.) %	Resene (av.) %
.1	Green	16.8	11.9	42.7	23.7	—	—	2.75	0.70	1.39
2	Green	16.6	17.1	32.8	26.0	—	—	2.49	0.63	1.26
3	Green	13.9	6.4	34.3	34.5	—	4.30	0.82	0.31	0.27
4	Green	16.4	5.9	38.0	30.0	—	—	3.10	0.79	1.57
8	Green	4.3	11.3	43.5	26.9	6.15	1.50	2.26	0.89	0.74
5, 6, 7, 9	Green	8.3	9.2	53.5	10.9	7.6	1.50	2.62	1.00	0.87
10	Green	6.4	7.15	54.3	22.7	6.2	4.30	0.82	0.31	0.27
11	Green	15.4	4.91	51.5	23.0	—	0.60	3.05	1.00	0.61
13	Seasoned	13.8	7.5	54.2	8.3	5.54	1.75	6.58	0.54	0.73

NOTE: (*a*) *The analytical data reported in Tables I to V are included in the above, being calculated on the basis of the weight of the total crude resin product as 100%.*
(*b*) *Where the products of two or more extractions were combined for analysis, the average percentages of the constituents for each extraction are given.*

Comments on Table VI

It will be noted that Extractions 8; 5, 6, 7, 9; 10 and 13 do not add up to 100%. This discrepancy is the result of an accumulation of small losses during the series of analyses. In the first place, average percentages are given in certain of the columns, since the individual amounts from each extraction were frequently too small for accurate individual analysis. In the second place, there were unavoidable losses of water soluble material during the various ether extractions, oxidations, etc.

The percentages given in column 3 are obtained from the initial analysis of the crude resins prior to the determination of the other individual constituents, so that losses in the subsequent analyses are not included.

With regard to note (a) it should be pointed out that certain values had to be recalculated on another basis so that any errors, averages, etc., are multiplied in the new figures.

It may be added that so far as the authors are aware, in none of the previous investigations where the crude resins were separated into the individual constituents has any attempt been made to account for a complete 100%.

Resin Acids

The crude resin acids, separated by preferential esterification from the fatty acids (Diagram I), were obtained in the form of a dark, brittle mass, resembling opaque rosin. The pure acids were isolated from the impure transformation products by three different methods: (a) treatment with petroleum ether, (b) vacuum distillation of the crude acids, and (c) vacuum distillation of the ethyl esters. The scheme of separation is shown in Diagram II.

Several methods were tried with the object of separating the pure resin acids from the impure mixtures. The only way in which it was found possible to do this without any apparent chemical change was to extract exhaustively with petroleum ether, and recrystallize repeatedly from aqueous alcoholic solution. In addition to the crystalline acids, certain amorphous, yellow acidic substances were obtained, which were very soluble in alcohol. These are possibly related to the sapinic acids. The residue insoluble in petroleum ether, when dried, formed a dark brown mass resembling very impure rosin. This residue probably consists of amorphous transformation products of the colophenic class, derived from the natural acids.

In the case of these resin acids, the constants such as iodine number, saponification value, ester number, melting point, etc., are very unreliable, as they vary considerably with the experimental conditions. This was found to be the case in the present investigation. The acid number, however, under the conditions employed in this investigation, is a direct measure of the true acidity of the pure substances, and offers the best means of determining the molecular weight accurately.

The molecular weight (302.8) of the acid obtained by petroleum ether extraction agrees with the theory for $C_{20}H_{30}O_2$ (302.2). The acids obtained by distillation, or esterification, were found to have a molecular weight of 309.0, possibly due to slight oxidation during the treatment. Carbon and hydrogen analyses in the past have led only to much confusion as regards the formula for these acids.

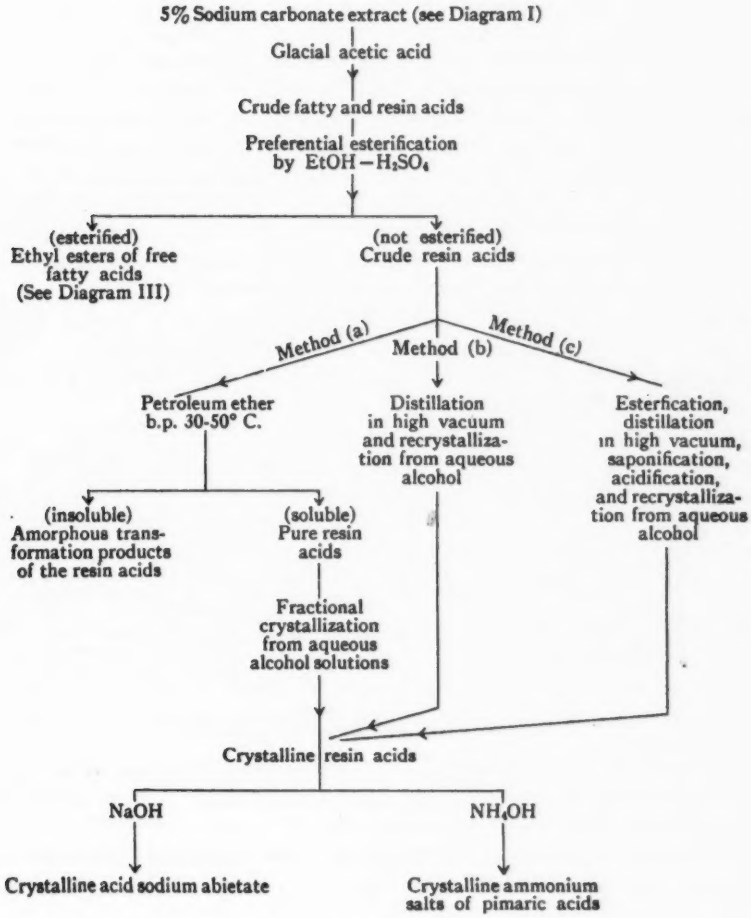
These products undergo isomerization near the melting point and always melt over a considerable range.

Identification of some of the individual resin acids has been made in the case of some oleoresins. This requires a considerable amount of raw material. Abietic acid has been estimated (16) in the presence of other resin acids by

means of the crystalline acid sodium salt $3(C_{20}H_{30}O_2).C_{20}H_{29}O_2Na$. The natural acids do not yield salts of this type. This salt was readily obtained in the present investigation, and the percentage of abietic acid thus calculated. The neutral sodium salt was gelatinous.

DIAGRAM II

METHOD FOR ISOLATION OF THE PURE RESIN ACIDS



A crystalline ammonium salt was obtained, indicating the presence of natural acids (pimaric group). Acids of the colophenic class yield only gelatinous ammonium salts (15, 26).

The higher yield of the acid sodium salt obtained from the acids which had been distilled or esterified, as compared with the acid isolated by extraction with petroleum ether, indicated that the material had been largely transformed to abietic acid during the treatment. The high yield of crystalline ammonium salt from the acids obtained by petroleum ether extraction showed that a considerable percentage of natural acids was still present in these products. Abietic acid is formed during the long series of recrystallizations, more ammonium salt being obtained from those acids which had received fewer recrystallizations. The purified acids obtained from the petroleum ether extraction in the present investigation were found to melt at 140-152°, 142-156°, 150-158°, and 151-162° C., after two, eight, fourteen and twenty recrystallizations respectively from aqueous alcohol. The final yield was about 1-2% of the original crude acids. When each of these lots was examined under the microscope, a mixture of at least two crystalline forms was observed. The apparently pure product consisted chiefly of needle-shaped crystals, with which was mixed a considerable percentage of the triangular crystals of abietic acid. It was impossible to separate them by mechanical means.

The total crude resin acids of green jack pine were found to contain approximately 66.3% crystalline acids, while the seasoned wood contained 51.8%; some of the pure products were probably transformed to amorphous substances during the seasoning process.

Certain other methods for the isolation of these pure acids, such as (a) fractional crystallization of the impure acids from organic solvents, (b) fractional precipitation of the impure acids from mixed solvents, (c) action of heat under pressure (15, 28), (d) purification by boiling with animal charcoal, (e) treatment with hydrochloric acid gas (19), (f) preparation of the phenylhydrazides (12), and the methylamides (23) were unsuccessful, on account of the presence of large amounts of impure, amorphous material, and consequent difficulty in isolating crystalline substances. The lead salts were also prepared (40, p. 653), but could not be isolated in a pure form; the portion soluble in alcohol, and that insoluble in alcohol, contained the same percentage of lead (33.0%); theory for $Pb(C_{20}H_{29}O_2)_2$, 25.6% Pb. The acids obtained by decomposing these salts were amorphous.

Distillation of the crude mixture of acids under high vacuum gave a small yield of crystalline products, a large proportion of the acids being transformed at the high temperature.

The method employed for esterifying the crude resin acid mixture was that especially adapted for esterification of dark rosin products (25), but evidently occasioned transformations of the acids obtained in the present research, due to the high temperature that had to be employed.

The resin acids could be removed from the wood in unchanged chemical condition by extracting at low temperatures with very low-boiling organic

solvents, but only small yields were obtained in this way. Also, various other substances were removed with the acids, thus necessitating a chemical separation in any case. Extraction with 1% aqueous alkali at a low temperature is inefficient, because a considerable quantity of lignin, in addition to fatty constituents, is removed also and the mixture is very difficult to analyse.

Experimental Part II

Isolation of Crystalline Resin Acids

Extraction with Petroleum Ether

The mass of crude resin acids (R, Diagram I) was thoroughly ground with a large quantity of clean quartz sand, and this mixture extracted in Soxhlet extractors with petroleum ether (b.p. 30-50° C.), until nothing further was dissolved. This required continuous treatment for six or seven days, with several re-grindings of the mixture. The extract was removed when it became concentrated, and replaced by fresh solvent, in order to avoid too prolonged heating of the dissolved acids. A pale-yellow solution having a green fluorescence was obtained, which yielded, on evaporation of the solvent, a yellow granular mass (acid A). The latter (A) was recrystallized once from 95% alcohol, and allowed to dry on porous tile. The mass was redissolved in boiling 95% alcohol, water carefully added until the solution was just turbid, the turbidity removed by heating, and the solution left to crystallize. These solutions were kept in vacuum desiccators, in which was placed a vessel containing calcium chloride, paraffin, or similar agent for absorbing the solvent. The crystalline mass thus obtained was treated several times in the same manner, but the products were still faintly yellow even after twenty recrystallizations. Other crops of crystals were obtained from the mother liquors, and purified in the same way.

The crystals were dried, first for three days over sulphuric acid, and then for one day at 100° C. under reduced pressure. The products were kept in amber-colored, sealed containers. The yield of crystalline acids, expressed as percentage of the total crude resin acids was; (a) from greenwood, 66.3%, and (b) from seasoned wood, 51.8%.

Vacuum Distillation of the Crude Resin Acids

The mixture of crude resin acids (R, Diagram I) was distilled at 0.01-0.05 mm. The initial fraction obtained below 180° C. was a yellow resin and yielded small amounts of crystalline material from alcoholic solutions. The higher fractions were very dark brown in color, and would not crystallize. At a bath temperature of 270° C. gaseous decomposition occurred, and the distillation was stopped.

The first fraction, when recrystallized from aqueous alcohol (as described above), yielded beautiful white needle-shaped crystals (acid B), which were dried over sulphuric acid under reduced pressure as already indicated.

TABLE VII

MEAN RESULTS OF SEVERAL DISTILLATIONS

Fraction boiling at ° C.	Pressure mm.	Distillate	Average yield on weight of crude resin acids %
1. 165-180°	0.01-0.05	Yellow resin	25
2. 180-210	0.01-0.05	Dark-brown resin, (brittle)	25
3. 210-250	0.01-0.05	Very dark, tarry substance	10
4. Residue, black tar			40

Purification of the Crude Resin Acids by Esterification

The mixture of crude resin acids (R, Diagram I) was esterified by the method of Johnston (25), and the reaction product distilled. The results are shown in Table VIII.

TABLE VIII

DISTILLATION OF THE ESTERIFIED CRUDE RESIN ACIDS

Esterification number	Distillation pressure, in mm.	Distillation temperature, ° C.	Yield of ester %	n_D^{22}
1	0.02–0.05	158–163	27.5	1.5440
2	0.02–0.05	161–168	33.5	1.5395
3	0.02–0.05	158–168	28.7	1.5520
4	0.02–0.05	156–163	49.1	1.5425

The ester distillate was saponified by boiling with an alcoholic solution containing four times the theoretical amount of potassium hydroxide. When isoamyl alcohol was substituted for ethyl alcohol, the yield of saponified product was practically the same.

The acid obtained by acidifying the saponification mixture with glacial acetic acid, extracting with ether, and evaporating, was recrystallized from alcohol with the addition of water, as already described, and colorless crystals obtained (acid C).

TABLE IX

RESULTS OF SAPONIFICATION

Product from esterification number	Saponification agent	Unchanged ester %	Saponified matter %
2	C_2H_5OH–KOH	25.4	74.6
4	Isoamyl alcohol-KOH	28.8	71.2

Preparation of acid sodium abietate (16). Solutions of each of the resin acids A, B and C in 95% ethyl alcohol were treated with one-quarter the theoretical amount of aqueous sodium hydroxide solution, calculated on the

TABLE X

SUMMARY OF PROPERTIES OF THE PURIFIED RESIN ACIDS IN JACK PINE

Acids from	Reference letter of acid used	Method of isolation	Number of times recrystallized from aqueous alcohol	M. p. ° C.	Iodine No.	Saponification No.	Acid No.	Esterification No.	Molecular weight	Abietic acids %	Pimaric acids %	Approximate quantity of crystalline acids in the crude acids %
Green wood extractions No. 1 to 11	B. C.	(B) Distillation (C) Esterification	8	151-161	90.9	239.0	181.5	57.5	309.0	88.6	—	66.3
Green wood extractions No. 1 to 11	A	Extraction with petroleum ether	20	150-162	129.2	243.4	185.3	58.1	302.8	—	56.8	
Seasoned wood extraction No. 13	A	Extraction with petroleum ether	2	140-152	(Not purified further)					Traces	68.2	51.8

basis of $C_{20}H_{30}O_2$ and the crystals dried at 60° C. under reduced pressure. Yield, from acids C and B (products of green wood) = 88.6%; m.p. 170-5° C. Acid A (from seasoned wood) yielded a gelatinous soap, with only traces of crystalline material.

Preparation of neutral sodium abietate. The acids A, B and C, in ethyl alcohol solution, were neutralized exactly with aqueous sodium hydroxide, and left to crystallize. The solutions yielded only gelatinous material.

Preparation of the ammonium salts of the resin acids. Alcoholic solutions of two lots of acid A, one from green wood, the other from seasoned wood, were mixed with a slight excess of concentrated aqueous ammonia. Most of the solvent was allowed to distil off very slowly in a vacuum desiccator, until the greater part had been removed. The precipitate was separated by filtration and washed on a porous tile with a little cold alcohol containing a few drops of ammonia. The product, after separation of the adhering gelatinous material in this manner, was recrystallized from alcohol containing ammonia, and was finally obtained in the form of colorless needles. It showed signs of decomposition at 115° C., and was completely liquified at 161° C. Klason and Köhler (26) found that this salt decomposed readily when heated, with loss of ammonia. Yield: (a) from acid A recrystallized twenty times (green wood) = 56.8%; (b) from acid A, recrystallized twice (seasoned wood) = 68.2% salt.

Fatty Constituents

The fatty constituents comprised free fatty acids and glycerides, or other esters. A systematic plan was developed for the investigation of each of these groups, permitting of their individual isolation, as outlined in Diagram III.

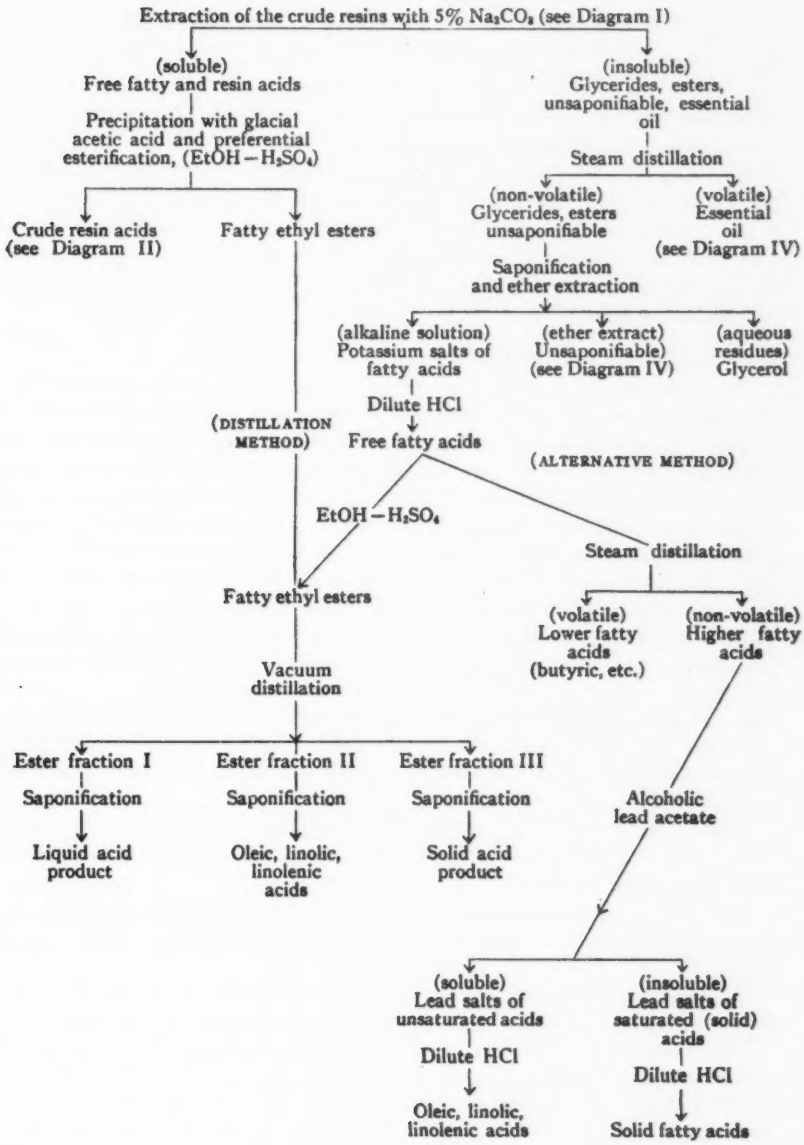
The glycerides and esters (Diagram III), when saponified and the saponified product acidified with HCl, yielded a mixture of acids of the saturated and unsaturated series. No esters of resin acids were found. The mixed ethyl esters of the fatty acids were prepared, and fractionally distilled to isolate the constituents. Another method employed involved the determination of the volatile acids by steam-distillation, and separation of the non-volatile portion into solid (saturated) and liquid (unsaturated) acids. After removing oxidized acids (insoluble in petroleum ether) from the solid products, the latter were subjected to fractional crystallization. The liquid acids were identified by their bromination or oxidation products. Constants such as iodine number, saponification value, refractive index, etc., were also noted.

The free fatty acids (Diagram III) present in the original resins were converted into their ethyl esters, and fractionally distilled at low pressure. Three constant-boiling fractions were always obtained, and these were examined separately.

The isolation of the individual esters was not possible by fractional distillation, as the boiling points were within a very close range, and constant-boiling mixtures were obtained finally. Repeated redistillation of the products resulted in considerable losses by decomposition and polymerization. A dark tarry residue always remained, which represented a considerable percentage of the material.

DIAGRAM III

METHODS FOR SEPARATION OF THE FATTY CONSTITUENTS



Volatile Acids

The procedure employed was based on the standard method of distillation of the acids with boiling water (30, p. 540). The amount of volatile material obtained was very small. Butyric acid was identified only by a qualitative test (17). Such a distillation probably involves a certain amount of oxidation of the non-volatile products.

Separation of Solid and Liquid Acids

The separation of the solid from the liquid acids by the relative solubilities of their lead salts in ether (31) is more difficult to carry out than the separation by the lead-salt-alcohol method (42); the latter is much more convenient, and is also just as accurate. The separation by either method is not strictly quantitative. The solid acid portion was always accompanied by some of the liquid acids, and vice versa, in the case of the products obtained in the present investigation.

Identification of the Individual Acids

The bromination experiments were always carried out on the mixtures of the free acids, and never on the esters; the brominated acid products are easier to identify, and have been studied more thoroughly. The new method of Steele and Washburn (39) for the quantitative estimation of the hexabromides was found to be more satisfactory than the older method of Eibner (18), although the procedure required greater care. The isolation of the bromination products of lower molecular weight than the hexabromides was found to be a difficult matter, since there is no satisfactory method for separating them, and their solubilities in various organic solvents are only relative. The process of fractional crystallization employed in the present investigation yielded only impure fractions of dibromide and tetrabromide, which could not be purified further on account of the small amount of material available.

A considerable amount of the oily dibromide of oleic acid was always obtained. The impure tetrabromide fractions melted between 95 and 116° C. The hexabromides were obtained in much purer form, and various specimens melted at 172-3°, 177-8°, and 180-3° C.

Melting points for similar products have been reported (30, vol. 1, pp. 202, 580) by some previous investigators as follows: tetrabromides, 113-4°, 114-5° C.; hexabromides, 181-2°, 180-1°, 177° C.

The quantitative determinations of the hexabromide indicated that linolenic acid is present in these fats in very small amount (less than 1% of the total fats).

The bromination products of the acids were readily soluble in chloroform, indicating that no unsaturated acids higher than linolenic were present.

The presence of oleic and linolic acids was confirmed by the fact that dihydroxy and tetrahydroxy (sativic) acids were obtained from the liquid acids by oxidation with permanganate. The two different forms of sativic acid (α and β) are said to correspond with two forms of linolic acid (30, pp. 237-38).

The results obtained in the bromination of the purified acid fractions, and of impure intermediate fractions, indicated that no other unsaturated acids besides oleic, linolic and linolenic were present in these fats.

The percentages of each of these unsaturated acids present in the mixture can be estimated from (a) the degree of unsaturation of the mixture; and (b) the amounts of the pure bromination products, although there are limitations to the use of either of these methods. In the present case, linolenic acid was estimated very accurately by the hexabromide determination in chloroform (39) but the other bromination products could not be isolated in sufficiently pure state for quantitative estimation. The percentages of oleic and linolic acid could be calculated approximately, however, from the unsaturation value, since the amount of linolenic acid present was already known.

Two acidic products found present in the fats in very small proportion could not be identified definitely. One of them was a colorless liquid, b.p. 55-60° C. at 0.005 mm. Carbon and hydrogen analyses of this ester corresponded to a formula $C_5H_9O_2$. The acid had a pleasant aromatic odor, and may possibly be derived from terpenes present in the wood, and removed with the fatty acids.

The other product was a solid white substance of waxy consistency which, when recrystallized, yielded a main fraction, m.p. 71-72° C. and two very small fractions from the mother liquors, m.p. 69-72° and 68.5-69.5° C., respectively. The latter possibly contained stearic acid, which melts at about the same temperature. The extremely small quantities of these products did not permit of further investigation. Sandquist (35) and Hasselstroem (22) obtained a similar solid acid, from Swedish pine oil, which melted at 72-73° C. The former considered it to be a dibasic lactonic acid having a molecular weight above 800. From saponification values of two impure fractions containing this acid obtained in the present investigation, the calculated molecular weights for the acid, if dibasic, would be from 714 to 1054, that is, of the same order as Sandquist's figure. This product is present to the extent of only about 2-3% in the total resinous material from the wood.

Experimental Part III

Glycerides and Fatty Acids in Jack Pine

(*a*) *Green wood*

Analysis of Fatty Glycerides

The glyceride and ester fractions (see Diagram III) from Extractions No. 5 to 10 (Table VI) were combined and saponified by heating for one hour with normal alcoholic potash. The unsaponifiable matter was removed by extraction with ether. The aqueous residue was acidified with dilute hydrochloric acid, extracted with ether, and the ether solution, after washing with water until the washings were neutral, dried over anhydrous sodium sulphate. After removal of the ether the acids thus obtained were separated by preferential esterification (46) with ethyl alcohol and sulphuric acid into (a) fatty

ethyl esters and (b) resin acids. Only traces of resinous impurities were found, so that all of the acidic constituents of this portion of the resins were of the aliphatic type.

The ethyl esters were fractionally distilled under low pressure, and the results are given in Table XI.

TABLE XI

DISTILLATION OF ETHYL ESTERS PREPARED FROM GLYCERIDES OF GREEN JACK PINE

Fraction No.	Pressure mm. mercury	Boiling point ° C.	Total esters %	Description	Temperature of constant-boiling mixture of carefully refractionated material ° C.
1	0.007-0.03	60-89	1.5	Mobile yellow liquid, pleasant odor	47-49 at 0.007 mm.
2	0.007-0.03	142-150	84.6	Viscous yellow liquid, fatty odor	135-137 at 0.007 mm.
3	0.007-0.03	155-188	2.5	Partly solid, yellow; fatty odor	156-158 at 0.007 mm.
Tarry residue, and volatile decomposition products.			11.4	Decomposed at 220° C.	

Fractions 1 and 3 (Table XI). The quantities obtained were very small; they were therefore added to the two similar ester fractions obtained from the glycerides (see below).

Analysis of constant-boiling mixture from Fraction 2 (Table XI): Boiling point, 135-7° C. at 0.007 mm., refractive index, $n_D^{20} = 1.4587$; iodine number, 130.3; saponification number, 185.6.

Bromination of the free acids (18, 30, p. 585): The products obtained were principally oleic dibromide; and linolic tetrabromide, m.p. 95-115° C.; also traces of linolenic hexabromide, m.p. 172-3° C.

Permanganate oxidation of the free acids (30, p. 575-76): The products were dihydroxystearic acid, m.p. 128-129°; α-sativic acid (recrystallized from water) m.p. 155°; β-sativic acid (recrystallized from alcohol) m.p. 173° C.

Approximate composition of fraction 2, Table XI calculated from the unsaturation value (2, p. 519; 30, p. 574): ethyl oleate, 41.7%; ethyl linolate, 58.3%; ethyl linolenate, traces. These figures, when calculated on the basis of the total esters (see Table XI) represent: ethyl oleate, 35.3%; ethyl linolate, 49.3%; ethyl linolenate, traces.

Analysis of Free Fatty Acids

The ethyl esters of the free fatty acids obtained from Extractions No. 5, 6, 7, 8, 9, and 10 (green wood) were fractionally distilled. The results are shown in Table XII.

Analysis of constant-boiling mixture from Fraction 1 (Table XII): Boiling point, 47-49° C./0.01 mm.; 180° at 1 atm., with decomposition; refractive index, $n_D^{20} = 1.4931$; iodine number, 30.3; saponification number, 182.0;

elementary analysis: C, 57.25, 57.37%; H, 9.48, 9.19%; empirical formula, $C_5H_9O_2$.

TABLE XII

FRACTIONAL DISTILLATION OF ETHYL ESTERS OF FREE FATTY ACIDS FROM GREEN JACK PINE

Fraction No.	Pressure mm.	Boiling point ° C.	Total esters %	Description	Temperature of constant-boiling mixture of carefully refractionated material ° C.
1	0.007-0.03	60-84	5.4	Mobile yellow liquid, pleasant odor	47-49 at 0.01 mm.
2	0.007-0.03	140-155	46.9	Viscous yellow liquid, fatty odor	135-137 at 0.01 mm.
3	0.007-0.03	159-185	24.6	Partly solid, yellow; fatty odor	156-160 at 0.01 mm.
Tarry residue, and volatile decomposition products.	0.007-0.03		23.1	Residue decomposed at 217°C.	

Analysis of constant-boiling mixture from Fraction 2 (Table XII): Boiling point, 135-8° C. at 0.01 mm., decomposes on heating in air; refractive index, $n_D^{20} = 1.4655$; iodine number, 117.5; saponification number, 173.5.

Bromination of the free acids. Products obtained were (a) oily dibromides, (b) linolic tetrabromide, m.p. 95-116° C.; (c) linolenic hexabromide,—two fractions, m.p. 172-3° C. and 180-3° C., in traces only. Approximate composition of Fraction No. 2 (2, p. 519, 30, p. 574): ethyl oleate, 57.1; ethyl linolate, 42.9%; ethyl linolenate, traces. Calculated on the total fatty ethyl esters (see Table XII) these figures represent: ethyl oleate, 26.8; ethyl linolate, 20.2%; ethyl linolenate, traces.

Analysis of constant-boiling mixture from Fraction 3 (Table XII): Boiling point, 156-60° C. at 0.01 mm.; decomposes when heated in air; m.p., 31-33° C.; refractive index, $n_D^{30} = 1.5410$; $n_D^{40} = 1.5335$; iodine number, 120.5; saponification number, 100.8. The acids from the saponification were separated (42) into: (a) solid fatty acids, 7.15%, (b) liquid fatty acids (by difference) 92.85%.

Bromination of the liquid-acid portion yielded the same products as from Fraction 2 above, namely, bromides of oleic, linolic, and linolenic acids.

TABLE XIII

EXAMINATION OF THE INTERMEDIATE FRACTIONS OF THE ETHYL ESTERS

Fraction, b.p. at 0.01 mm. ° C.	Iodine number	Saponification number	Bromides from the free acids
60–120	81.7	234.0	Oily dibromide only
130–135	89.7	127.0	Oily dibromide only
130–150	—	141.5	Oily dibromide, traces of hexabromide
140–165	—	207.6	All three bromides

The solid acid fraction represents about 2.2% of the total ester mixture (Table XII). The product was purified by recrystallization from alcohol; three fractions were obtained, m.p. 71-72°, 69-72°, and 68.5-69.5° C. respectively.

These fractions are mixtures of the three main constant-boiling mixtures, Table XII. The above results indicate that oleic, linolic and linolenic are the only unsaturated acids present.

(*b*) *Seasoned Wood*

Analysis of Fatty Glycerides

The fraction containing the glycerides and esters from Extraction No. 13 (Table VI) was saponified by boiling for one hour with alcoholic potash and the unsaponifiable matter was removed by ether extraction (see Diagram III).

Glycerol was determined (2, p. 658, 30, p. 453) in the aqueous liquor from the saponification. Yield of glycerol: 0.39% of original weight of crude resins.

Analysis of the Acids from the Saponification Mixture

The saponification product (Extraction No. 13) was acidified with hydrochloric acid (see Diagram III) and the crude mixture of volatile and non-volatile fatty acids extracted with ether. The ether extract was washed free of mineral acid and dried over anhydrous sodium sulphate. Evaporation of the ether left a dark brown semi-liquid mass weighing 24.5 grams.

(*a*) *Determination of Volatile Acids* (*30, p. 540*)

The aqueous distillate from the steam distillation of this product (24.5 gr.) required 21.90 cc. of 0.1004 *N* NaOH for neutralization. A qualitative examination of the product (17) indicated the presence of butyric acid only; 21.90 cc. of 0.1004 *N* NaOH = 0.1936 gm. butyric acid = 0.79% of the total acids from the above glycerides.

(*b*) *Non-volatile Acids.*

These were recovered by ether extraction of the steam distillation residue obtained above (a). The ethereal extract was dried over anhydrous sodium sulphate, evaporated, and the residue separated (42) into solid and liquid fatty acids; the oxidized acids were removed from the solid fraction. Composition: liquid acids, 48.7; solid acids, 20.8; oxidized acids 30.2%. The solid acid fraction was recrystallized from alcohol several times, and a white solid product, m.p. 71-72° C. finally obtained, in very small amount. This was added to the same material previously isolated.

Analysis of the Liquid Acid Fraction

Iodine number, 119.4: bromination products (39); hexabromide, 3.01% and 2.82%, corresponding with 1.10 and 1.03% linolenic acid; mean = 1.07% of acid.

Dibromide and tetrabromide fractions were obtained from the mother liquors, as before. Approximate composition (2, p. 519; 30, p. 574) of the liquid acid fraction: oleic acid 69.00; linolic acid, 29.93; linolenic acid 1.07%.

Calculated on the total original acids from the glycerides, these figures represent approximately: oleic acid, 33.6; linolic acid, 14.6; linolenic acid, 0.5%, together with the volatile and solid acid portions already mentioned.

Analysis of the Free Fatty Acids

Ethyl esters of the free fatty acids from Extraction 13 (seasoned wood) were fractionally distilled and the results are shown in Table XIV.

TABLE XIV

FRACTIONAL DISTILLATION OF ETHYL ESTERS OF FREE FATTY ACIDS FROM SEASONED WOOD

Fraction No.	Pressure mm.	Boiling point ° C.	Total esters %	Description	Number of times redistilled
1	0.007-0.01	50-65	5.2	Nearly colorless liquid: pleasant odor	Once
2	0.007-0.01	125-150	43.5	Pale yellow; viscous; fatty odor	Once
3	0.007-0.01	150-170	25.8	Pale yellow; partly solid; fatty odor	Once
Tarry residue, and volatile decomposition products			25.5		

Analysis of constant-boiling mixture from Fraction 1 (Table XIV): Boiling point, 50-65° C. at 0.01 mm.; refractive index, $n_D^{20} = 1.4940$; saponification number, 51.2; the amount of acid recovered from the saponification mixture was too small to permit of identification of the product.

Analysis of constant-boiling mixture from Fraction 2 (Table XIV): Boiling point, 125-150° at 0.01 mm.; refractive index, $n_D^{20} = 1.4732$; iodine number, 127.8; saponification number, 157.0.

Bromination of the acids (39). Hexabromide (average), 1.00%, equivalent to 0.36% linolenic acid. Other fractions containing dibromide and tetrabromide were also obtained, as before.

Approximate composition (2, p. 519; 30, p. 574) of Fraction 2: ethyl oleate, 45.1; ethyl linolate, 54.5; ethyl linolenate, 0.36%. Calculated on the total ethyl esters (see Table XIV) these figures represent: ethyl oleate, 19.6; ethyl linolate, 23.7; ethyl linolenate, 0.16%.

Analysis of constant-boiling mixture from Fraction 3 (Table XIV): Boiling point, 150-170° C. at 0.007-0.01 mm.; refractive index, $n_D^{30} = 1.5481$; $n_D^{40} = 1.5355$; saponification number, 103.2; the acid from the saponification mixture was purified from alcohol and found to be the same solid acid previously obtained, m.p. 71-72° C.

Analytical Methods

Iodine number (3) was determined by means of Wijs' iodine chloride solution.

Saponification number (3). Alkali Blue 6B indicator was used in conjunction with phenolphthalein, on account of the dark color of the saponifica-

TABLE XV

THE FATTY CONSTITUENTS OF JACK PINE RESINS. SUMMARY OF ANALYTICAL RESULTS

Kind of wood	Product examined	Unsaturated acids approximate % of each acid	Main ester fractions %			Iodine numbers of ester fractions			Saponification numbers of ester fractions			Volatile acids %	Liquid acids %	Solid acids %	Oxidized acids %
			1	2	3	1	2	3	1	2	3				
Green	Glycerides and esters	Oleic, 35.3 Linolic, 49.3 Linolenic, traces	1.5	84.6	2.5	—	130.3	—	—	186	—	—	—	—	—
Green	Free fatty acids	Oleic, 26.8 Linolic, 20.2 Linolenic, traces	5.4	46.9	24.6	30.3	117.5	120.5	182	173	101	—	—	—	—
Seasoned	Glycerides and esters	Oleic, 33.6 Linolic, 14.6 Linolenic, 0.5	—	—	—	—	119.4	—	—	—	—	0.79	48.7	20.8	30.2
Seasoned	Free fatty acids	Oleic, 19.6 Linolic, 23.7 Linolenic, 0.16	5.2	43.5	25.8	—	127.8	—	51.2	157	103	—	—	—	—

tion mixtures. The acids were recovered in each case, for further examination, by extracting the acidified solution with ether, washing the ethereal solution with water, drying over fused Na_2SO_4, and evaporating.

Acid number (3). $N/20$ Alcoholic potash was used, in a microburette, and the acids were recovered as described above.

Separation of solid from liquid fatty acids. This was effected by the lead-salt-ether method (31) and the lead-salt-alcohol method (42). The acids were examined as described below.

Isolation of oxidized acids. The fraction containing the solid fatty acids isolated by the lead-salt-alcohol method was dried at 60° C. under reduced pressure, and thoroughly ground with a large quantity of clean sand. The mass was extracted with petroleum ether (b.p. 30-50° C.) in a Soxhlet, until no more was dissolved. The petroleum ether solution yielded the pure solid acids, which were then subjected to fractional crystallization from alcohol.

The residue, insoluble in petroleum ether and consisting of oxidized and polymerized fatty substances of acidic nature, was not investigated further.

Bromination of the unsaturated fatty acids. Eibner's method (18) and also Steele and Washburn's method (39) were used. After separation of the insoluble hexabromide, and removal of excess bromine (by thiosulphate (30, p. 583) or amylene (39)), the ethereal solution containing the lower bromination products was evaporated, and the residue fractionally crystallized from petroleum ether (b.p. 30-50° C.). After numerous recrystallizations, two main fractions were obtained, consisting of dibromide and tetrabromide respectively.

Permanganate oxidation of the unsaturated acids (30, pp. 575-76). The chief products obtained, in addition to lower fatty acids, resulting from decomposition of the higher members, were (a) dihydroxy stearic acid, m.p. 128-9°; (b) α-sativic acid, m.p. 155°; and (c) β-sativic acid, m.p. 173° C. The yields of products by this oxidation process are never quantitative.

Experimental Part IV

Essential Oil

The essential oil was obtained by steam-distillation of the residue from the crude resins after removal of the acidic constituents, etc. (see Diagram IV). The purified product was a colorless oil possessing the odor of turpentine. Further fractionation was not possible on account of the smallness of the amount of the purified product. The substance readily polymerized on heating.

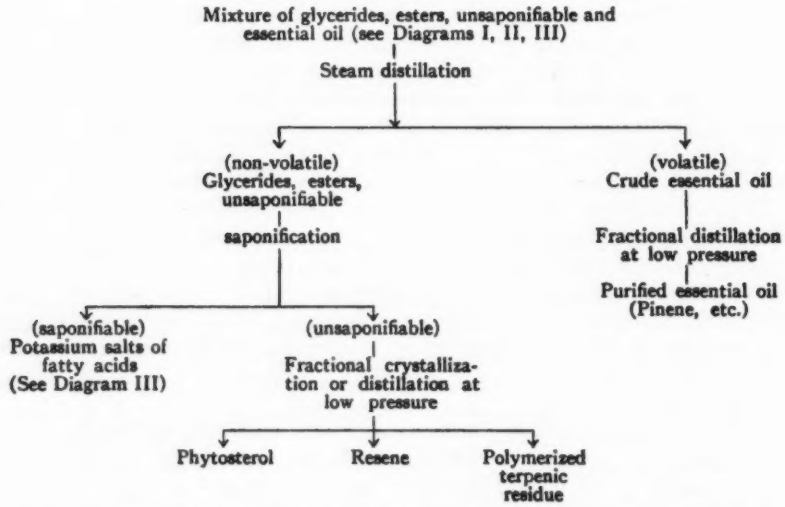
Boiling point, refractive index, etc., indicated that the oil from the seasoned wood was identical with that from the green wood. The yield was very small in both cases, and no variation of amount of oil with time of seasoning could be observed. The product was found to be highly dextrorotatory, as in the case of American turpentine essence.

The oil yielded an impure nitrosochloride, which was very difficult to isolate, this being in harmony with the high optical rotation of the oil.

The results pointed to the presence of α-pinene, while tests for camphene, sylvestrene, dipentene, limonene, and β-pinene, gave only negative or indefinite results. On account of the small amount of pure material available and the difficulties in carrying out some of these reactions, it cannot be assumed, without further confirmation, that these terpenes were absent.

DIAGRAM IV

SEPARATION OF THE ESSENTIAL OIL AND UNSAPONIFIABLE MATTER



The crude essential oil obtained by steam-distillation was purified by repeated distillation under very low pressures. The product tended to polymerize when heated at atmospheric pressure.

TABLE XVI

PROPERTIES OF ESSENTIAL OIL PURIFIED BY DISTILLATION AT LOW PRESSURES

Properties	Oil from green wood	Oil from seasoned wood
Boiling point at 0.01 mm., ° C.	59–63	59-62
Boiling point at 760 mm., ° C.	156–158	159–160
Refractive index	$n_D^{20} = 1.4874$	$n_D^{20} = 1.4879$
Specific gravity at 20° C.	0.9289	0.9330
$[\alpha]_D^{24}$	+39° (c = 1.242 in alcohol)	

Tests for Individual Constituents of the Essential Oil

α-Pinene was identified by the preparation of the hydrochloride (20), nitrosochloride (5, 43, p. 251), nitrol-piperidine (43, p. 253), and nitrosopinene (43, p. 252).

TABLE XVII
YIELD OF ESSENTIAL OIL

Kind of wood	Resin extraction No.	Essential oil in total crude resins %
Green	3, 10	4.30
Green	5, 6, 7, 8, 9	1.50
Seasoned	13	1.75

The hydrochloride and nitrosochloride were obtained in very small yield (1-2%). The amount of the nitrosochloride available was so small that the products obtained in the preparation of the nitrol-piperidine and nitrosopinene could not be purified sufficiently for complete identification.

β-Pinene: Identification was attempted by means of alkaline permanganate oxidation (44), and the usual mixture of oxidation products was obtained. A white solid portion, probably containing nopinic acid, was finally isolated in very small quantity, insufficient for complete purification. The melting point was indefinite.

Tests for camphene, sylvestrene, dipentene and limonene (5, p. 1447; 7), made on very small amounts of the pure material, yielded either inconclusive or negative results. No solid dihydrochlorides or tetrabromides could be obtained, even after standing for about three weeks.

UNSAPONIFIABLE

The unsaponifiable matter (isolated as indicated in Diagram IV) from the crude resin mixture consisted of a light brown, waxy mass, various specimens of which melted between 65 and 68° C. Two principal methods were used in the investigation of this material: (1) fractional crystallization; and (2) fractional distillation.

Fractional Crystallization

Various fractions removed by crystallization from an alcoholic solution of the unsaponifiable matter were purified by repeated recrystallization from alcohol or acetone. Three separate products were finally obtained, (a) a white crystalline product, m.p. 131-2° C., identified as one of the phytosterols; (b) an inert, waxy material (resene); and (c) a mass of polymerized terpenic material which could not be further separated.

The complete separation of the sterol from the resene was accomplished by means of the addition compound which it forms with digitonin. The sterol was also identified by yielding an acetate, m.p. 125-6° C. and gave the characteristic color reactions for the sterols.

The resene could not be saponified at high temperature and pressure, and therefore it was not a wax.

Fractional Distillation at Very Low Pressures

Both liquid and solid products were obtained. The small amounts of liquid consisted of essential oil which had not been removed in the steam-distillation

previously. The higher-boiling solid fractions were subjected to fractional crystallization, and three products obtained, corresponding exactly with the three constituents (a), (b) and (c) mentioned above.

The separation of the individual constituents of the unsaponifiable matter by the two methods just mentioned was found to be prolonged and tedious. Complete separation of the sterol from the amorphous resene either by means of the acetate or the digitonide was found to be difficult, and required considerable time. Fractional distillation even at the low pressures employed was found to cause decomposition of a considerable portion of the material.

The process of fractional crystallization from alcoholic solution, followed by purification of the products from alcohol or acetone, without preparing the acetate or digitonide, seems to be the quickest and most efficient means of separating the constituents.

The residue of polymerized terpenic material (c) mentioned above resembles the material left after distillation of turpentine oil. A higher percentage (83.9%) of this product was found in the unsaponifiable matter from seasoned wood as compared with that from green wood (60.3%), this being probably due to changes taking place in the oils present in the wood during the time of seasoning. The yields of sterol and resene were also found to be appreciably smaller in the case of seasoned wood.

Experimental Part V

Method for the Investigation of the Unsaponifiable

The scheme finally used is indicated in Diagram IV.

Fractional Crystallization

The unsaponifiable matter was dissolved in about fifteen times its weight of 95% boiling alcohol, and allowed to stand for several days at room temperature. The precipitate of very fine needle crystals was filtered off. Upon cooling the mother liquor in the ice-box, a second precipitation, consisting of some of the crystalline product mixed with amorphous material, was obtained and separated by filtration. Three further precipitations were obtained by evaporating some of the solvent each time, cooling, and filtering. A small amount of residual solution remained which yielded no further solid material, and this was evaporated under reduced pressure, leaving a very viscous mass of polymerized terpenic material. The latter had a softening range between 9-25° C., and could not be completely solidified even at −15 to −20° C. When distilled for a long time with steam, only traces of volatile turpentine oil were obtained. The material was very dark brown in color, and had a refractive index $n_D^{20} = 1.5600$.

The five solid precipitates mentioned above were subjected separately to a lengthy series of fractionations from alcoholic solutions, by which means the crystalline material was separated from the amorphous. The latter was concentrated in the mother liquors from the various fractions. The needle-like crystals melted at 131-2° C. and upon treatment with acetic anhydride (1)

yielded an acetate, m.p. 125-6° C. The fractions containing the mixture of crystalline and amorphous material could be completely separated by treatment with digitonin (45, 27) and decomposition of the resulting complex formed, the latter being a characteristic property of the sterol alcohols. The crystals gave the usual color reactions of the sterols (2, pp. 770-71).

The amorphous fraction of the unsaponifiable matter could not be effectively purified from alcoholic solutions on account of the gelatinous nature of the precipitates. Acetylation (1), followed by fractional crystallization from alcohol or acetone, was found to have no advantage, since the product did not differ appreciably in solubility from the original material. It was finally purified by treatment with digitonin as follows:

One part of the mixture of the sterol and amorphous material was dissolved in one hundred parts hot 95% alcohol, and about one-fourth of its volume of a boiling 1% solution of digitonin in 95% alcohol added slowly with stirring. The solution was then allowed to stand at 0° C. overnight, and the precipitate, consisting of the complex formed by the digitonin and the phytosterol, filtered off. The above treatment was repeated until no further precipitation was obtained.

The combined precipitates were washed with ether, and then decomposed as described below. The alcoholic mother liquors (a) contained the amorphous material (resene) together with the excess digitonin.

The digitonin complex was suspended in a paper thimble immediately above the surface of boiling xylene, in a flask fitted with reflux condenser, and refluxed for 10 hr. The digitonin remained in the thimble, while the purified sterol was obtained by removal of the xylene with steam-distillation, and recrystallization of the residue from alcoholic solution. The pure sterol melted at 132° C.

The alcoholic mother liquors (a), containing the resene, were evaporated, and the residue treated with boiling xylene exactly as described above, thus removing the excess digitonin. Removal of the xylene by steam-distillation of the xylene solution left a semi-solid product, which was purified by solution in alcohol and evaporation, yielding a pure white resene of waxy consistency, various specimens of which melted between 62 and 68° C.

The resene was heated at 140° C. for eight hours in a sealed pyrex tube, with twenty times its weight of saturated alcoholic sodium hydroxide. The cooled mixture was transferred to a separatory funnel, an equal volume of saturated salt solution added, and then extracted with petroleum ether (b.p. 30-50° C.) until all soluble material had been removed.

The petroleum ether extract yielded a yellow residue, which was dried on porous tile, recrystallized twice from alcohol, and finally dried at 50° C. in vacuo, m.p. 65-66° C. It was apparently identical with the original resene.

The aqueous layer from the saponification mixture was acidified, and extracted with ethyl ether. The ethereal extract yielded only a trace of dark brown material.

Fractional Distillation of the Unsaponifiable Matter

The crude unsaponifiable matter was fractionally distilled at pressures below 1 mm. using a Langmuir pump. Five fractions were collected and the distillation was stopped when the bath temperature reached 320° C. on account of gaseous decomposition.

TABLE XVIII

FRACTIONAL DISTILLATION OF CRUDE UNSAPONIFIABLE MATTER

Fraction	Boiling point ° C.	Pressure mm.	Distillate	Percentage by weight
1	60–100	0.01	Nearly colorless liquid	3.1
2	170–177	0.01	Viscous, greenish-yellow liquid	7.8
3	177–185	0.01	Pale-yellow solid	22.0
4	185–220	0.01	Dark-yellow solid	31.6
5	220–270	0.01	Dark-yellow viscous liquid	13.7
Residue in flask				19.2
Losses (by difference)				2.6
				100.0

When purified by redistillation, Fraction 1 was found to consist of some essential oil not previously removed in the steam-distillation of the total unsaponifiable matter. Fraction 2 probably consisted of some polymerized terpenic material formed by the action of heat on the essential oil. There was not enough to enable a thorough examination to be made.

The other three products were crystallized fractionally from petroleum ether solutions, and in this way the solid materials separated from the dark-brown oily mass of polymerized terpenic substances. The latter had the same properties as the similar material already mentioned as having been isolated in the process of fractional crystallization of the unsaponifiable matter.

No further fractionation of the solid materials could be effected by means of petroleum ether, or by fractional precipitation with mixtures of petroleum

TABLE XIX

UNSAPONIFIABLE MATTER

Wood used	No. of extraction of unsaponifiable matter	Method used in examination	Composition of unsaponifiable matter		
			Sterol %	Resene %	Polymerized terpenes %
Green	1, 2, 4	Fractional crystallization	14.4	28.8	56.8
Green	3, 5, 6, 7, 8, 9, 10	Fractional crystallization	22.0	19.4	58.6
Green	11	Fractional crystallization	21.3	13.2	65.5
		Mean value	19.2	20.5	60.3
Seasoned	13	Fractional crystallization	5.8	10.2	84.0
Seasoned	13	Fractional distillation	7.9	8.3	83.7
		Mean value	6.9	9.3	83.9

ether of different boiling points, so the products were subjected to a lengthy series of recrystallizations from 95% alcohol. The final products were (a) a white, crystalline substance, m.p. 131-2° C. having properties identical with those of the sterol previously mentioned, and (b) an amorphous resene, m.p. 62-69° C., the same as that isolated previously.

A complete summary of the analytical results for the unsaponifiable matter from various batches of crude resins is given in Table XIX.

Conclusion

The methods devised for the present investigation were primarily for the isolation and identification of the various constituents. Any thorough investigation having for its object the elucidation of the chemical changes which take place in these resins with time and exposure during seasoning would necessitate the development of other technique, beyond the scope of the present investigation. From the results obtained, however, it would seem that the differences in the character of the two classes of resins, from green and seasoned jack pine respectively, are associated with the changes taking place in the character and amount of certain constituents, such as the unsaturated fats and fatty acids, on the one hand, and with the changes in the resin acids, on the other. The former contain readily oxidizable and polymerizable substances of the unsaturated type, such as oleic, linolic and linolenic acids, while the latter represent a group of products characterized by marked instability under the influence of light, heat and oxidizing agents. The role played by the easily polymerizable terpenic products in yielding polymerized products of a sticky, tacky nature should be noted.

As regards the bearing which these changes may have on the efficient manufacture of sulphite pulp, the question as to whether any of these are facilitated by the action of solutions of sulphurous acid and bisulphites at an elevated temperature cannot be answered until the necessary experiments involved in such a research have been carried out. It seems possible, however, that marked changes in one or more of the constituents may take place under such conditions. On the other hand, the opportunity afforded the resinous constituents of green wood during storage for a year or more on the wood pile possibly provides a time period and environment in which the less stable constituents of the fats, fatty and resin acids are converted into more inactive, polymerized substances of such a character as to show a certain degree of inertness under the conditions involved in the manufacture of wood pulp.

The question of pitch trouble in the manufacture of paper is associated to a notable degree, as pointed out by Campbell (11), with the temperature and hydrogen ion concentration of the pulp mixture containing the resinous products in the form of an emulsion or colloidal suspension.

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CONDUCTIVITY DATA OF AQUEOUS MIXTURES OF HYDROGEN PEROXIDE AND ORGANIC ACIDS[1]

By W. H. Hatcher[2] and M. G. Sturrock[3]

Abstract

Preliminary results obtained by measuring changes in conductivity of organic acids mixed with aqueous hydrogen peroxide show a rapid though measurable progression to the attainment of a maximum or minimum within an hour. These establish previous findings, and afford a clue to the conductivity of organic peracids.

Introduction

During the course of investigations dealing with the oxidation of organic acids by means of hydrogen peroxide, it was found that in nearly every case the rates of oxidation suggested the formation of a complex which later underwent re-orientation and disruptive oxidation (2, 3). Attempts to identify this complex gave results indicative of the formation of peracids,—a process requiring time for the establishment of an equilibrium according to the general equation:—

$$R.COOH + H_2O_2 \leftrightarrows R.COOOH + H_2O.$$

It was at first thought that the peracid was the complex indicated and that this was a step in the oxidative process. More recent results, however, suggested that the peracid may be only incidental to the progress of oxidation.

Besides confirming and amplifying previous findings regarding the peracids of formic, acetic, and propionic acids (1), similar compounds were discovered with glycollic, isobutyric, lactic, and maleic acids, together with indications of similar formations with malonic, malic and tartaric acids.

The following pages describe an attempt to ascertain what conductivity changes occur on adding hydrogen peroxide to aqueous solutions of some simple organic acids.

Experimental

Solutions of formic, acetic, propionic and glycollic acids were made up with water and hydrogen peroxide and kept at 0.5° C.; the concentrations and temperature were such as were known not to involve any oxidation to lower substances or loss of reagents, and conformed with those formerly studied for purposes of comparison.

The apparatus used was that of Maass and Cuthbertson (4), and later used by the authors in the investigation of dihydroxy maleic acid. The only modification necessary was in the dimensions of the cells which were made to conform to the differences in conductivity of the acids used.

The preparation for conductivity measurements involved weighing the reagents separately, cooling to 0.5° C. and then thoroughly mixing. The readings were made as soon thereafter as possible.

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The following tables give the data connected with the four acids studied.

TABLE I

RESISTANCE OF FORMIC ACID IN AQUEOUS HYDROGEN PEROXIDE

Reagent	Concentrations	
	Per cent	Gram-mols.
Formic acid	14.07	0.032
Hydrogen peroxide	10.44	0.032
Water	75.49	

Time in min.	Resistance in ohms	Time in min.	Resistance in ohms
15	42,097	60	43,333
20	42,441	165	43,478
25	42,660	225	43,552
30	42,801	500	43,552
40	43,095	660	43,567

TABLE II

RESISTANCE OF GLYCOLLIC ACID IN AQUEOUS HYDROGEN PEROXIDE

Reagent	Concentrations	
	Per cent	Gram-mols.
Glycollic acid	22.55	0.032
Hydrogen peroxide	10.40	0.033
Water	67.05	

Time in min.	Resistance in ohms.	Time in min.	Resistance in ohms.
20	73,733	95	75,070
25	74,000	120	74,913
30	74,411	135	74,560
35	74,624	150	74,492
40	74,798	155	74,420
50	74,912	21 hr.	73,724
60	74,982	68 hr.	73,724

TABLE III

RESISTANCE OF ACETIC ACID IN AQUEOUS HYDROGEN PEROXIDE

Reagent	Concentrations	
	Per cent	Gram-mols.
Acetic acid	27.71	0.0490
Hydrogen peroxide	10.07	0.0314
Water	62.22	

Time in min.	Resistance in ohms	Time	Resistance in ohms
15	270,720	19 hr.	268,260
30	271,060	2 days	268,260
95	269,090	18 days	268,260
135	268,790		

TABLE IV

RESISTANCE OF PROPIONIC ACID IN AQUEOUS HYDROGEN PEROXIDE

Reagent	Concentrations	
	Per cent	Gram-mols.
Propionic acid	43.13	0.5824
Hydrogen peroxide	9.90	0.2912
Water	46.97	

Time in min.	Resistance in ohms.	Time in days	Resistance in ohms.
15	907,900	7	887,580
25	907,900	14	887,580
120	887,580		

Conclusions

The most striking observation which results from a consideration of the data given in Tables I, II, III and IV is that in all four cases the conductivity changes to a constant value within four or five hours. The change is rapid at first and then gradually slower.

In the case of formic acid the conductivity of the solution decreases to a constant level within four hours. Using solutions of the same concentration, Hatcher and Holden (3) have found that the reaction mixture attains equilibrium in 30 hr. with the production of 3.78% performic acid, as determined by chemical analysis. This can only be explained by assuming the formation of some complex which is not the peracid, but which later rearranges to form the peracid. Moreover, the complex and the peracid must have the same conductivity.

With the glycollic acid the same rapid decrease in conductivity within one hour occurs, followed by a gradual increase and finally the attainment of a constant value in about 30 hr. Using solutions of the same concentration Hatcher and Holden (3) have found that the production of perglycollic acid is not complete until six days have elapsed from the time of mixing the reagents.

Acetic acid behaves in the same general way as glycollic. There is a sudden sharp decrease in conductivity followed by a gradual increase. In both cases the final conductivity is found to be very slightly greater than that of the solution immediately after mixing.

Propionic acid, however, behaves in a different manner. There is a sudden increase in conductivity which becomes constant within three hours.

To explain the foregoing results completely will require further experimentation along similar lines. However, the object of attacking this problem has been attained, for in each of the four cases studied, where data on the rate of reaction implied the formation of a complex, the addition of hydrogen peroxide brought about a rapid though not instantaneous change in conductivity of the organic acid. This change ultimately reached a fixed value when some peracid was undoubtedly present; this constancy implies that the conductivity of the peracid in each instance is of the same order as that of the complex. No previous data on the conductivity of organic peracids are available, but it is hoped that it may be possible to secure such in the near future.

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CALCIUM RELATIONSHIPS OF FORAGE CROPS[1]

By L. I. Pugsley[2] and R. R. McKibbin[3]

Abstract

The calcium content of timothy and red clover hays was found to increase directly as the total calcium content of the clay loam soils in which they were grown, and to decrease as the "lime requirement" of these soils increases. There is no proportionate increase of protein nor of phosphorus as the calcium content of the hays increases.

The timothy hays from the area investigated are extremely low in calcium content but are otherwise normal. The ratios $\frac{CaO}{P_2O_5}$ and $\frac{protein}{CaO}$ for these timothy hays are, respectively, abnormally low and abnormally high. These values appear to be normal in the red clover hays.

Introduction

In an investigation of the calcium and phosphorus content of some Quebec red clover and timothy hays, carried out by Holcomb (7), wide variation in calcium composition was noted. The work reported in the present paper was planned with the object of determining the causes of this variation, and of extending the field of observation.

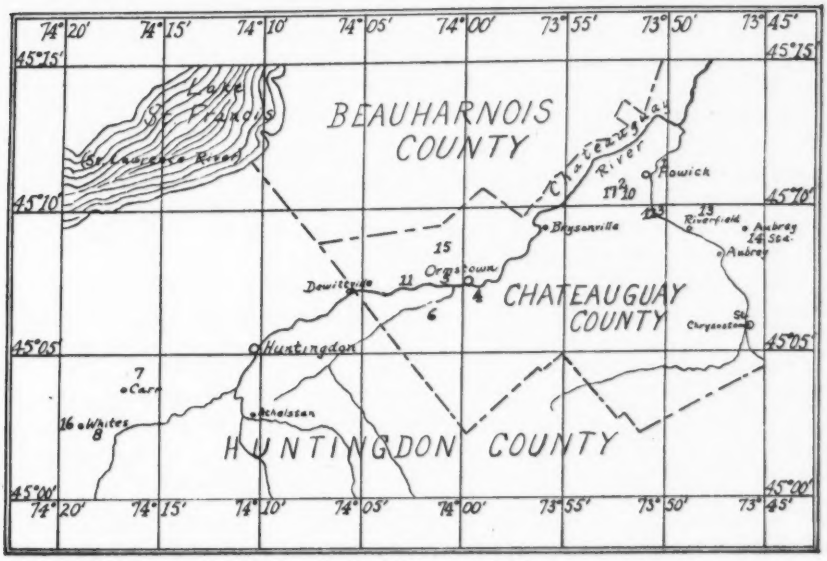

Fig. 1. *Key map showing where red clover, timothy and soil samples were taken.*

[1] *Manuscript received October 17, 1930.*
Contribution from the Department of Chemistry, Macdonald College, with financial assistance from the National Research Council of Canada.

[2] *Research Assistant.*

[3] *Assistant Professor of Chemistry, Macdonald College.*

On July 8, 9 and 10, 1929, the authors, conducted by Professor Alex. R. Ness of Macdonald College, visited the region shown in the map (Fig. 1) and secured from representative farms all the soil and hay samples examined in this investigation, except those from location No. 9. This dairy farming district from which nearly all the samples were taken is one of the finest in Canada. In general, the samples were collected on farms lying along the banks of the Chateauguay River, and the soils were heavy clay loams. None of these soils has ever received lime or commercial fertilizer treatment, but all have been heavily fertilized with barnyard manure. In this Chateauguay River basin eight samples of surface soil, eight of subsoil, 13 of timothy and 11 of clover hay were secured on the dates mentioned. On July 13, 1929, one sample of surface soil, one of subsoil, one of timothy and one of red clover hay were taken at location No. 9 at the west end of Montreal Island.

In collecting the hay samples the stems of the growing timothy and clover plants were cut off individually with scissors, 1 to 2 in. above the surface of the ground. The timothy plants were just past first bloom, while the red clover was in full bloom with no brown heads showing. The hay samples when cut were placed in 24-lb. paper bags for transportation. Especial care was taken to ensure that no blooms or leaves were lost. Immediately on arrival at the laboratory, on July 10, the samples from the Chateauguay district were removed from the paper bags and allowed to dry in an atmosphere free from fumes. The hay samples from location No. 9 were placed to dry the day they were cut.

The soil and hay samples were collected at the same time, the former from the identical soils in which the timothy and clover plants were growing. To ensure obtaining representative samples of the soils composite samples of both surface and subsoil were taken. At eight different points scattered over the area from which the growing forage crop plants were taken, holes were made in the ground and subsamples secured.

Surface-soil samples were obtained by cutting with a spade clean slices one inch thick from the freshly exposed side of each of the eight holes. The eight samples thus obtained were thoroughly mixed on a rubber sheet and from the mixed contents a composite sample of from 8 to 10 lb. weight was taken for transportation to the laboratory. Subsoil samples were taken at a depth of from 16 to 24 in. Composite samples of subsoil were obtained in the same manner as with the surface soil; namely, by cutting a slice from the clean side of each of the eight holes at the proper depth, mixing thoroughly and finally taking from 8 to 10 lb. of the mixture to the laboratory.

Analytical Methods

(*a*) *Soils*

The soil samples were placed in clean tin coffee cans with tin covers for transportation. As soon as the soils reached the laboratory they were tested for moisture content, and, while still moist and fresh, for *p*H value. The samples were then air-dried, and sieved through a 1-mm. mesh screen, the

portion passing through being ground finely in an iron mortar with an iron pestle, and then bottled pending further analytical work.

The methods employed in analysing the soils are described in detail elsewhere (10). In practically all cases the methods used were those of the Association of Official Agricultural Chemists (1). The *p*H values of the soils were determined electrometrically, organic carbon by a modification of Read's method (14) and total sulphur by the magnesium nitrate fusion method of Swanson and Latshaw (15).

(*b*) *Hays*

The methods used by Holcomb (7) were adopted for the preparation of the samples and for the moisture and ash determinations. The pulverization of the samples, however, was effected in a Wiley mill. Protein (1, p. 116, No. 8 and p. 8, No. 22) was determined by the method of the A.O.A.C. Calcium was determined by the method used by Holcomb (7).

Magnesium nitrate fusion methods for phosphorus were found unsatisfactory with the hays on account of the explosive nature of the dried mixture of magnesium nitrate and hay. The explosions occurred when the samples

TABLE I

ANALYSIS OF SOILS AND SUBSOILS

Soil No.	Initials of farmer	Moisture when sampled %	*p*H of fresh moist soils	Lime requirement (lbs. CaO per acre)	Hygroscopic moisture of air-dried sample %	Ratio $\frac{C}{N}$	Ratio $\frac{SiO_2}{Al_2O_3+Fe_2O_3}$
1	A.B.	14.77	5.39	3930	3.71	10.8	2.57
		13.35	5.61	2200	4.76	6.53	2.22
2	R.R.N.	15.77	6.10	3960	2.65	14.3	3.11
		15.68	6.78	1280	4.80	9.44	2.24
3	J.E.N.	15.47	5.35	2710	2.87	10.5	2.98
		16.77	6.35	1700	4.76	9.77	2.28
4	J.M.W.	14.53	6.05	2440	2.22	12.7	3.71
		10.89	5.71	1600	2.30	9.65	2.61
5	J.H.	20.22	5.90	1480	2.17	13.6	3.22
		17.44	6.56	980	2.14	9.25	2.92
6	P.F.	14.79	6.47	2260	3.77	12.0	2.90
		10.72	5.65	1140	2.91	9.61	2.64
7	G.M.	18.85	5.81	4085	3.72	12.1	2.88
		17.97	5.25	2425	5.19	10.8	2.28
8	W.L.C.	16.24	8.00	800	3.15	12.1	3.54
		15.32	7.23	875	2.42	6.76	3.10
9	M.C.	16.62	6.00	1780	2.02	10.9	3.50
		11.37	5.95	1390	1.17	9.56	3.46

NOTE:—*Subsoil data given directly below soil data.*

TABLE II

ANALYSIS OF SOILS AND SUBSOILS

Soil No.	Nature of soil	CaO %	MgO %	K_2O %	Organic C %	N %	SO_3 %	P_2O_5 %
1	Grey-brown clay loam	1.78 1.77	1.69 2.44	2.19 2.33	3.03 0.44	0.28 0.07	0.13 0.05	0.28 0.19
2	Grey-brown to blue clay loam	1.85 2.02	3.06 3.18	2.02 2.42	3.60 0.48	0.25 0.05	0.10 0.04	0.21 0.19
3	Medium brown clay loam	1.85 2.06	1.12 1.50	2.29 2.68	1.84 0.39	0.17 0.04	0.09 0.04	0.20 0.16
4	Grey-brown to white clay loam	1.74 2.01	1.46 2.06	2.13 2.34	2.61 0.41	0.21 0.04	0.09 0.02	0.25 0.17
5	Grey-brown to white clay loam	2.39 2.95	1.45 1.85	2.38 2.74	2.34 0.38	0.17 0.04	0.07 0.04	0.17 0.19
6	Grey-brown to white clay loam	2.38 2.28	1.59 1.88	1.81 1.98	3.39 0.31	0.28 0.03	0.13 0.03	0.30 0.20
7	Grey-brown to blue clay loam	1.25 1.35	1.69 2.23	2.02 2.32	3.24 0.54	0.27 0.05	0.11 0.06	0.32 0.19
8	Grey-brown to white clay loam	2.35 2.13	1.12 1.78	2.31 2.72	3.51 0.30	0.29 0.04	0.14 0.05	0.29 0.25
9	Grey-brown clay loam with fine whitish sand.	2.21 2.33	1.55 1.58	1.74 1.85	2.96 0.54	0.27 0.07	0.11 0.07	0.23 0.18

NOTE:— *Subsoil data given directly below soil data.*

TABLE III

COMPOSITION OF TIMOTHY HAY

Sample No.	Year after seeding	Nature of crop	Initials of farmer	Ratio of $\frac{\text{protein}}{\text{CaO}}$	Ratio of $\frac{\text{CaO}}{P_2O_5}$
1	2nd	Poor	A.B.	73	0.227
2	1st	Medium	R.R.N.	204	0.078
3	1st	Good	J.E.N.	215	0.075
4	1st	Good	J.M.W.	189	0.112
5	1st	Poor	J.H.	93	0.204
6	1st	Good	P.F.	95	0.374
7	1st	Good	G.M.	220	0.092
8	1st	Good	W.L.C.	113	0.148
9	1st	Good	M.C.	102	0.157
10	2nd	Poor	J.W.	129	0.160
11	1st	Medium	W.R.	86	0.292
12	1st	Good	A.M.	242	0.064
13	1st	Medium	R.T.	128	0.099
14	1st	Good	A.A.	199	0.102

were placed on the hot plate for further drying, *i.e.*, before they were put in the muffle furnace for ignition. The use of a smaller sample of hay for this method was considered unsatisfactory for gravimetric determinations because of the small amount of phosphorus present. The alternative method for phosphorus, mentioned by Holcomb but not used by him, proved satisfactory. This method involved the digestion of a 5-gm. sample of hay in a Kjeldahl flask, with concentrated sulphuric acid and 2 to 4 gm. of sodium nitrate (1, p. 2, No. 6 (d)). The results given in this paper are all based on this method.

Results

The results of analysis of soils and hays are given in Tables I to VI, inclusive.

TABLE IV

COMPOSITION OF TIMOTHY HAY

Sample No.	Percentage on moisture-free basis				
	Moisture	Ash	CaO	P_2O_5	Protein (N×6.25)
1	7.32	6.16	0.089	0.396	6.52
2	7.35	5.63	0.034	0.442	6.97
3	7.24	4.15	0.033	0.488	7.25
4	6.66	5.37	0.040	0.362	7.68
5	7.36	6.97	0.075	0.370	7.02
6	7.37	5.26	0.053	0.144	5.15
7	6.34	6.50	0.028	0.314	6.35
8	7.29	5.59	0.071	0.478	8.12
9	7.34	5.48	0.063	0.406	6.49
10	7.63	5.71	0.053	0.332	6.86
11	6.66	6.11	0.083	0.287	7.26
12	6.83	6.17	0.033	0.524	8.15
13	6.34	6.50	0.049	0.500	6.35
14	6.81	5.78	0.032	0.314	6.36

TABLE V

COMPOSITION OF RED CLOVER HAY

Sample No.	Year after seeding	Nature of crop	Initials of farmer	Ratio of $\frac{\text{protein}}{\text{CaO}}$	Ratio of $\frac{\text{CaO}}{P_2O_5}$
1	2nd		No red clover sample taken		
2	1st	Medium	R.R.N.	9.80	4.66
3	1st	Good	J.E.N.	12.22	3.20
4	1st	Good	J.M.W.	12.31	3.25
5	1st	Poor	J.H.	8.12	4.80
6	1st	Good	P.F.	6.96	8.00
7	1st	Good	G.M.	23.16	1.38
8	1st	Good	W.L.C.	8.49	4.77
9	1st	Good	M.C.	12.80	3.44
10	2nd		No red clover sample taken		
11	1st	Poor	W.R.	8.67	5.10
12	1st	Good	A.M.	12.25	3.25
13	1st	Medium	R.T.	8.85	5.20
14	1st	Good	A.A.	8.48	4.48

TABLE VI

COMPOSITION OF RED CLOVER HAY

Sample No.	Percentage on moisture-free basis				
	Moisture	Ash	CaO	P_2O_5	Protein (N×6.25)
1	No red clover sample taken				
2	7.79	6.91	1.53	0.328	14.95
3	8.36	6.99	1.35	0.422	16.50
4	10.07	5.66	1.43	0.440	17.60
5	8.02	6.06	1.85	0.385	15.03
6	8.52	6.09	1.91	0.239	13.26
7	9.11	7.13	0.65	0.479	15.01
8	8.32	6.15	1.57	0.328	13.30
9	7.02	6.60	1.10	0.320	14.08
10	No red clover sample taken				
11	9.29	7.75	1.66	0.325	14.40
12	7.78	7.02	1.18	0.364	14.46
13	8.71	6.15	1.60	0.309	14.16
14	8.31	6.15	1.72	0.383	14.58

Discussion

Parker and Truog (13), using very largely analytical results reported by Henry and Morrison (6) and by Wolff (16), pointed out that calcium in plants increases more regularly with increasing nitrogen than any of the other elements. They divided the important agricultural plants into two groups; (a) those having a low Ca:N ratio and a low lime requirement, (b) those having a high Ca:N ratio and a higher lime requirement.

Dr. J. D. Newton (11) pointed out that the composition of soils upon which the plants were grown were unknown to Parker and Truog and it may have happened that most of the plants containing high percentages of calcium were grown on soils rich in calcium. Newton found that barley and vetch, or barley and pea plants did not absorb nitrogen and calcium from water cultures in proportional amounts.

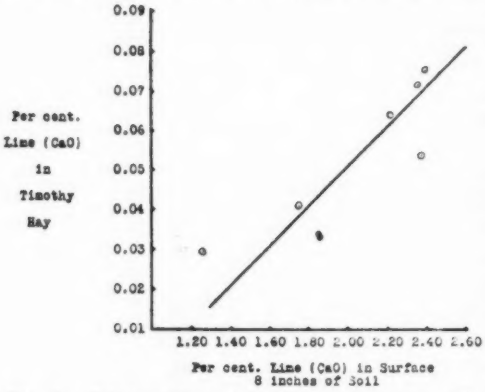

FIG. 2. *Relation between per cent lime in 9 surface soils and 9 timothy hays grown on these soils in 1929.*

In the results given in the tables and figures in the present investigation it will be noted that the timothy and clover plants have not been found to absorb nitrogen and calcium from the soils in proportionate amounts. It has been found, however, as shown in the tables and in Fig. 2 and 3, that the lime percentage in the hays increases proportionately with the percentage of lime in the surface soils. The protein percentage

in the hays does not show a proportionate increase with the lime. As illustrated in Fig. 6, 7, 8, 9, 10 and 11, the ratio of protein to lime in both clover and timothy hays decreases as the percentage of lime in the hays, as well as in the soils, increases. This indicates that the increase in lime in the hays is independent of that of the protein.

Available calcium was not determined in the soils. As previously observed, however, these soils have not, during their cropping history of a century or more, received any treatment with lime or commercial fertilizer. Consequently the lime requirement values and their total calcium oxide content should be a good indication of the "lime level" of the soils. The lime requirement values of the surface soils vary inversely as the lime content of both timothy and clover hays as shown in Fig. 4 and 5, which confirm the evidence presented by Fig. 2 and 3.

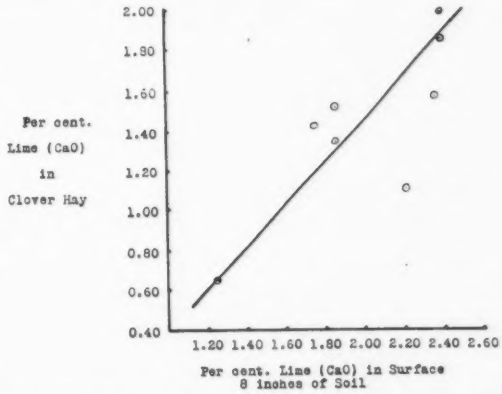

FIG. 3. *Relation between per cent lime in 8 surface soils and 8 red clover hays grown on these soils in 1929.*

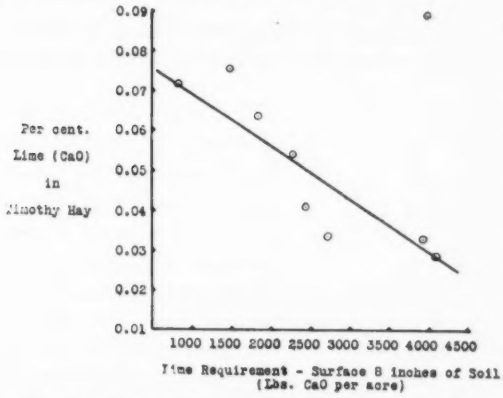

FIG. 4. *Relation between lime requirement of 9 surface soils and the per cent lime in 9 timothy hays grown on these soils in 1929.*

The extremely low lime content of the timothy hays from these soils is interesting. The clover hays appear to be normal in this respect. In Table VII are presented the results of analyses of red clover and timothy hays reported from a number of different sources, for lime, phosphorus and protein, and ratios existing in the hays.

The data presented in Table VII show that the red clover hays from this district are normal, while the timothy hays are very low indeed in their calcium content. The ratios of lime to phosphorus and of protein to lime illustrate this.

In Fig. 12 and 13 the fact is shown that as the percentage of lime in both clover and timothy hays increases the ratio $\frac{CaO}{P_2O_5}$ does not remain constant

but likewise increases. This means that when there is a low percentage of lime in the hays (and in the clay loam soils), there is more phosphorus present in the hays, relative to lime, than when a greater percentage of lime is present.

TABLE VII

LIME, PHOSPHORUS AND PROTEIN CONTENT OF HAYS

Analysts or compilers of data	CaO % (av.)	P_2O_5 % (av.)	Crude protein (N×6.25) % (av.)	Ratio protein/CaO (av.)	Ratio CaO/P_2O_5 (av.)
Forbes, E. B., *et al.* (4)					
Red clover	1.599	0.387	13.00	8.13	4.13
Timothy	0.248	0.259	5.34	21.53	0.95
Orr, J. B., (12)					
Red clover	1.60	0.39	—	—	4.10
Timothy	0.25	0.31	—	—	0.81
Parker, F. W., and E. Truog (13)					
Red clover	2.41	0.664	14.88	6.17	3.63
Timothy	0.574	0.733	8.50	14.81	0.78
Holcomb, R. (7)					
Red clover	1.508	0.366	—	—	4.12
Timothy	0.100	0.273	—	—	0.37
Pugsley, L. I., and R. R. McKibbin					
Red clover	1.461	0.310	14.78	10.12	4.29
Timothy	0.053	0.380	6.89	130.00	0.14

Both timothy and clover plants appear to take up increasing quantities of lime more readily than increasing quantities of phosphorus; the phosphorus percentage varies less.

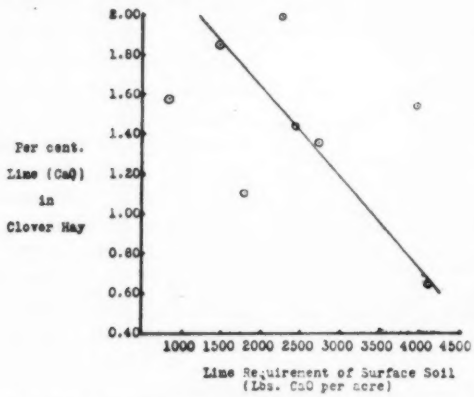

FIG. 5. *Relation between lime requirement of 8 surface soils and the per cent lime in 8 red clover hays grown on these soils in 1929.*

This phenomenon is undoubtedly related to the supplying power of the soil. It is a well known fact that soils normally contain a greater amount of calcium available to plants than they do of all other basic ions combined. The phosphate ion, in which form phosphorus is assimilated by plants, is in the soil solution in a very small quantity at any time.

No direct relationship is evident between the per cent protein in the timothy and clover hays and the total

nitrogen, calcium oxide, lime requirement or *p*H values of the surface soils, nor is any direct relation evident between the total phosphorus pentoxide or *p*H value of the soils and the phosphorus pentoxide content of the hays.

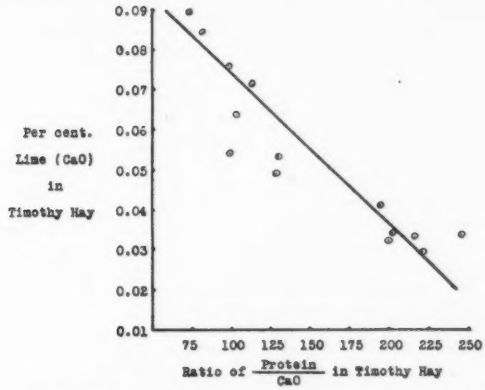

FIG. 6. *Relation between per cent lime and the ratio* $\frac{protein}{CaO}$ *in 14 timothy hays in 1929.*

Fonder (3) found that the quantity of calcium in the soil solution, the soil texture and the soil reaction all greatly influence the calcium content of pea plants, which vary greatly in this respect in different soils.

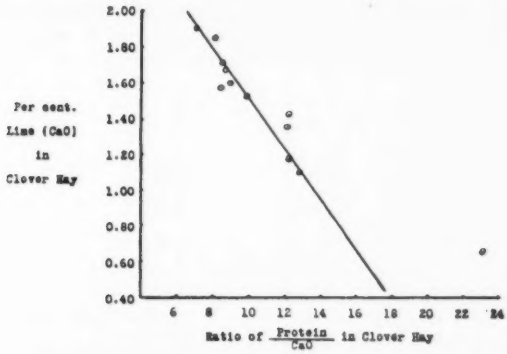

FIG. 7. *Relation between per cent lime and the ratio* $\frac{protein}{CaO}$ *in 12 red clover hays in 1929.*

The presence of lime in the rations of animals is of great importance. Henderson and Weakley (5) found that dairy animals fed on rations which were low in calcium, or in calcium and phosphorus, grew about as well as normally-fed animals for some time, but at the end of two years had not attained the

same weight or height at withers. Animals fed on rations low in phosphorus grew as well as those receiving normal rations over a two-year period, even though the inorganic phosphorus in the blood was below normal. The bones

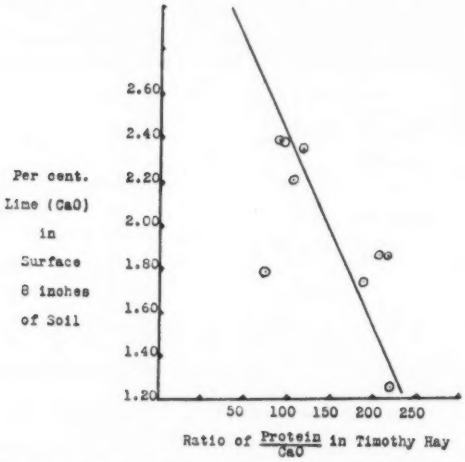

FIG. 8. *Relation between per cent lime in 9 surface soils and the ratio* $\frac{protein}{CaO}$ *in 9 timothy hays grown on these soils in 1929.*

of animals fed on rations with less than 0.35% of calcium or 0.20% of phosphorus were low in ash although, regardless of the ration used, the $\frac{CaO}{P_2O_5}$ ratio in bone remained the same.

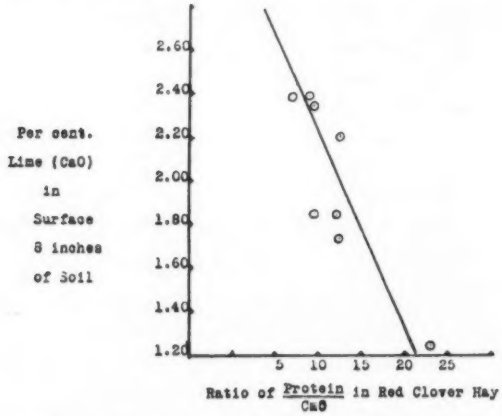

FIG. 9. *Relation between per cent lime in 8 surface soils and the ratio* $\frac{protein}{CaO}$ *in 8 red clover hays grown on these soils in 1929.*

Bethke, *et al.*, (2) and Massengale (8) emphasize the importance of a proper $\frac{CaO}{P_2O_5}$ ratio in rations for pigs and chicks.

In this province a large amount of feed is purchased for dairy cattle. Because

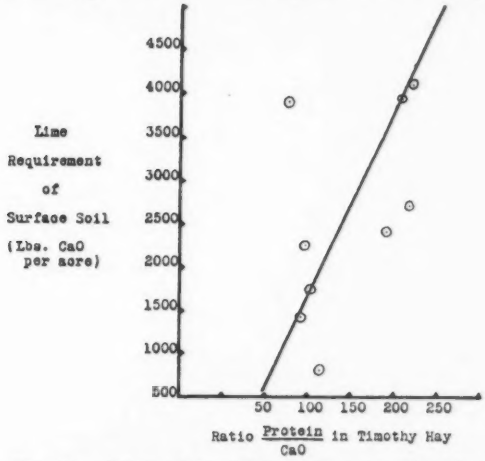

FIG. 10. *Relation between lime requirement of 9 surface soils and the ratio* $\frac{protein}{CaO}$ *in 9 timothy hays grown on these soils in 1929.*

of the importance of the lime factor in roughage feeds, and because of the great variations in this respect existing at present, it would appear that liming of the heavily-cropped soils of the dairy farms of Quebec province must be practiced to a much greater extent in the future than it is at present.

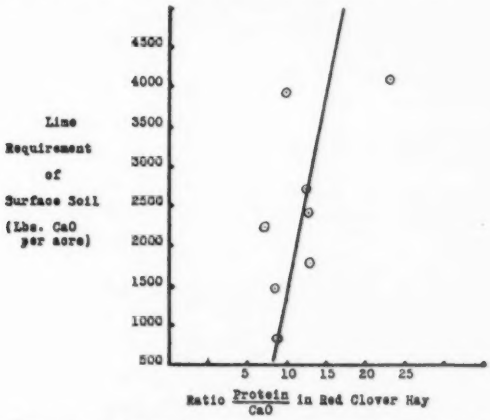

FIG. 11. *Relation between the lime requirement of 8 surface soils and the ratio* $\frac{protein}{CaO}$ *in 8 red clover hays grown on these soils in 1929.*

The phosphorus content of both timothy and red clover hays from this Chateauguay River basin area appears to be satisfactory. Mather (9) has shown that by fertilization with superphosphate fertilizers the percentage of inorganic phosphorus in forage crops may be greatly increased where necessary.

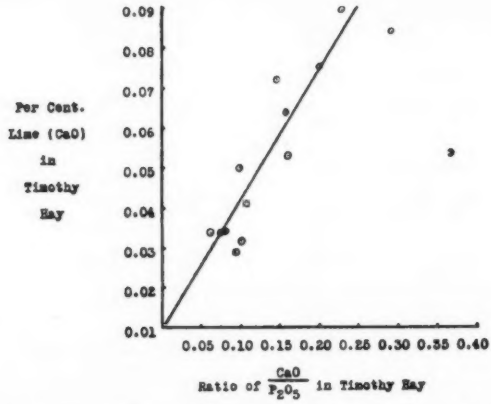

FIG. 12. *Relation between per cent lime and ratio* $\frac{CaO}{P_2O_5}$ *in 14 timothy hays in 1929.*

The question of quality of forage crops, as evidenced by chemical analyses, is an important one, particularly with regard to mineral constituents. Commercial concentrated feeds are generally much higher in acidic than in basic

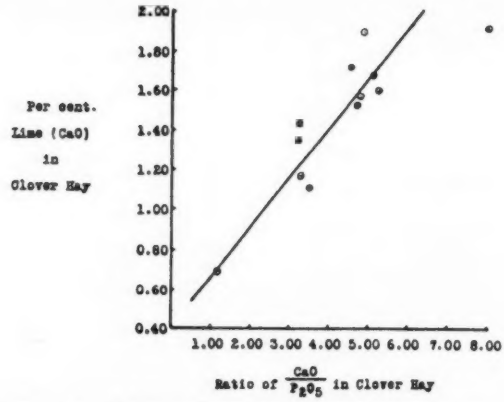

FIG. 13. *Relation between per cent lime and ratio* $\frac{CaO}{P_2O_5}$ *in 12 red clover hays in 1929.*

ash constituents and the stock feeder must rely largely upon his roughage feeds or supplementary mineral feeding to supply the base minerals. It is believed

that other criteria are at least as important in determining the value of forage feeds as is the total yield of dry matter per acre, although the latter measure of crop response, which is the one almost exclusively in use at the present time, is the most obvious criterion.

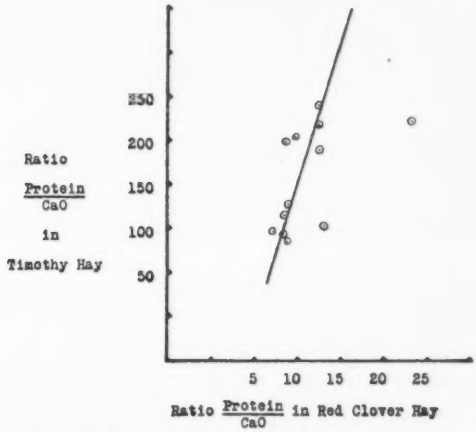

FIG. 14. *Relation between ratio* $\frac{protein}{CaO}$ *in 12 red clover and 12 timothy hays grown in the same soils in 1929.*

Acknowledgments

The authors wish to acknowledge the assistance of Professor Alex. R. Ness, who helped to secure the samples used in this investigation, and of Mr. W. R. Waugh who prepared the hay samples and made all of the analyses for moisture and ash and some of those for protein.

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NOTE ON A PHENOMENON CONNECTED WITH THE AURORA[1]

By A. C. Burton[2]

Abstract

A narrow and distinct arch of light was observed to stretch across the sky overhead from East to West, remaining steady in position for some forty minutes. A photograph, on microscopic examination, showed an unexpected fine structure that was not observed visually.

There is a remarkable phenomenon of comparatively rare occurrence connected with the aurora that has been particularly evident this summer in Canada, and which seems to deserve attention and record.

The particular observation was made on August 21, 1930, at a place about 75 miles southwest of Ottawa (Latitude 44°45′N, Long. 76°30′W), some 15 miles from any town. At about 10 p.m. on a very clear moonless night attention was drawn to a brilliant white beam of light which stretched right across the heavens from East to West overhead, intersecting the Milky Way at right angles. Its angular width appeared to be not more than 2°, and it was so bright and well-defined that were it not for its extent it might have been thought to be a searchlight beam. The highest point of the arch, which seemed to be part of a circle round the earth, was south of the observer's zenith, at an angle of about 70° to the Pole Star.

Preceding the phenomenon there had been a brilliant display of aurorae of the usual 'streamer' type in the North, but when the arch described was seen at its brightest these were entirely absent. The remarkable beam remained steady for some 40 min. before fading slowly. In places it was observed to be made up of a series of streaks parallel to the arch but slightly displaced laterally relative to each other, in 'echelon'.

A camera, of focal length 7.5 in., the only one available at the time, was pointed at the beam, a little to the west of overhead, and the shutter left open for 25 min. On the resulting film there was a remarkably fine and well-defined line, which shows exactly the characteristics noted visually. Its definiteness and narrowness, when contrasted with the tracks made by adjacent stars (the bright star to the right-hand side of the print is Arcturus), show how fixed in position in the atmosphere the phenomenon must have been during the 25-min. exposure. Examination of the negative under a microscope revealed that the 'streak' had an unexpected fine-structure of parallel lines, in places seven or eight in number, giving the appearance of a diffraction pattern. Plates *A*, *B*, *C* and *D* are from microphotographs (magnification 45 times) of the line at the places indicated on the plate above. The width of these lines approaches in some cases the limit of resolution of the camera, as estimated from the width of the star-tracks. If we suppose that the phenomena are at a height of

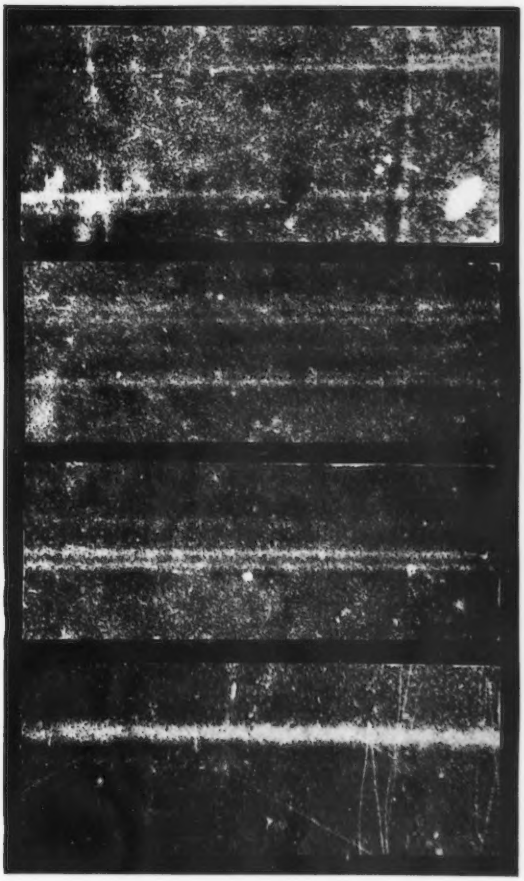
[1] *Manuscript received November 18, 1930.*

Contribution from the Physical Laboratory of the University of Toronto, Toronto, Canada, with financial assistance from the National Research Council of Canada.

[2] *Research student, University of Toronto.*

PLATE I

A B C D



A

B

C

D

Photographs of the phenomenon observed. A, B, C and D are from microphotographs (magnification 45 times) of the line at the places indicated on the plate above.

500 miles, the two bright lines in *C* are about 400 yd. apart and are accurately parallel for 100 miles.

Though the beam appeared visually to be fairly narrow and definite, nothing of this structure was apparent. The explanation would seem to be, either that the fine lines are due to transient flashes which, however, were not noted at all and would scarcely be so orderly in arrangement, or that the different color sensitivity of the photographic plate from that of the eye reveals these narrow sources of light within the broader, more diffuse, white beam.

The same phenomenon of a white arch light across the sky was noted by Mr. J. Allen, at Winnipeg on the same date, and on August 30 at 9 p.m. a similar appearance was observed by the author in Ontario, though it was fainter and lasted only 10 min. Similar displays have been remarked upon last summer in Northern Ontario.

It is of interest to know how these distinct and narrow beams of light are produced so far south of the Pole. From the photograph using the star tracks and known altitude of Arcturus we find that the angle of the beam to the Pole Star at the observer is about 60°—so that the latitude of the beam must have been at most less than that of the place of observation (45°). There was increased activity of sunspots near the date in question; (we are at the maximum of the cycle), though there seem to have been no remarkable magnetic changes at the time.

GAS-METAL ELECTRODE POTENTIALS IN STERILE CULTURE MEDIA FOR BACTERIA[1]

By Eldon M. Boyd[2] and Guilford B. Reed[3]

Abstract

The work of French and Kahlenberg on gas-metal electrode potentials suggested that the potentials obtained at inert metal electrodes immersed in growing cultures of bacteria, and generally regarded as an oxidation-reduction phenomenon, might be of a similar type.

This has been tested by passing air, hydrogen, nitrogen and carbon dioxide through sterile beef broth and observing the potentials developed at platinum, gold, and mercury electrodes. It is shown that air causes a rise in potential at all three electrodes; hydrogen causes a marked fall in potential at the platinum and a slight change at the gold and mercury electrodes; nitrogen and carbon dioxide cause little or no change in the potential at the three electrodes. These changes are in agreement with French and Kahlenberg's results except in the case of the mercury electrode which is shown to react chemically with the broth.

Introduction

An inert metal such as gold or platinum placed in a growing culture of micro-organisms registers, when connected with a standard half-cell in the usual potentiometer circuit, a difference of potential which becomes more negative as growth proceeds. This is generally regarded as an oxidation-reduction phenomenon. Voluminous data have been accumulated during the last few years concerning the chemical and physical aspects of oxidation-reduction potential changes, but little information is available to account for the potential change occurring in a growing culture of micro-organisms. The principal theories advanced have been concerned with the accumulation of reducing substances such as the sulphydryl bodies or with the removal of oxygen by respiration. Scant attention has been given to other metabolic processes in this connection.

French and Kahlenberg (11) have shown that polished inert metals form gas-metal electrodes with gases, and that the potentials are specific for a particular metal and gas. Much experimental evidence is presented to show that the gas-metal electrode potentials, developed when such metals are immersed in solutions of potassium chloride and exposed to the gases, result from the absorption of the gas by the metal and the formation of surface gas films. Since many organisms produce gases such as hydrogen, carbon dioxide, and hydrogen sulphide during growth, the question arises: do such gases act on inert electrodes in bacteriological media as they do in the potassium chloride solutions used by French and Kahlenberg? A correlation between gas production and potential change has recently been clearly indicated by Lepper and Martin (14). They show that low potentials are obtained in cultures of organisms which produce sulphides or hydrogen. Gillespie (12) noted that

[1] *Manuscript received October 31, 1930.*

Contribution from the Department of Bacteriology, Queen's University, Kingston, Ontario, Canada.

[2] *Graduate student, Queen's University.*

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cultures of *B. coli* in glucose broth attained negative potentials of the order of that of the hydrogen electrode. Cannan, Cohen and Clark (2) obtained similar results and briefly discussed the possible action of hydrogen. These findings have been further confirmed by other investigators (1, 3).

The present paper is concerned with the effects of such gases on inert metal electrodes in sterile culture media.

Methods

The medium used was beef extract broth except in one experiment, as noted, in which beef infusion broth was substituted. The broth was prepared in the usual way, buffered with 0.1 *M* di-sodium phosphate and adjusted to *p*H 7.3. In the earlier experiments the medium was autoclaved in the electrode vessels, sterile bright metal electrodes inserted, gas inlet tubes carried to the bottom of the vessels and the half-cells thus made connected by means of sterile, saturated potassium chloride-agar bridges with a saturated potassium chloride-calomel electrode. After a cell was rapidly assembled in this manner the broth was covered with a layer of sterile vaseline and potential readings begun at once. In later experiments, the freshly autoclaved broth was immediately covered with a layer of vaseline and allowed to stand at room temperature for a week. The electrodes and connections were then inserted through the vaseline layer. It was found that broth aged anaërobically in this way, cooled to 0° C. and maintained at this temperature during the course of the experiment, gave much less variation in potential. In both procedures the gas to be tested was allowed to flow in at the rate of about 25 to 30 bubbles per minute; a faster rate caused little or no alteration in the final potential attained although it affected slightly the speed at which this potential was reached.

The effects of four gases, hydrogen, nitrogen, carbon dioxide and air were investigated with platinum, gold and mercury electrodes. The platinum and gold electrodes consisted of polished foil in glass tubes. The gas inlet tubes were carried to the bottom of the vessels so that the gas was admitted just below the electrodes. For mercury electrodes about 5 cc. of pure mercury was placed in the bottom of the electrode vessel, broth was added above the mercury and, when ready, the gas to be investigated was forced under the surface of the mercury. Commercial tank nitrogen, hydrogen and carbon dioxide were used and air was blown in by means of an electric pump. In certain experiments, as described, the gas was carefully purified.

Readings of e.m.f. were obtained by a Leeds and Northrup potentiometer and portable galvanometer. The latter instrument permits considerable polarization at the electrode when the circuit is closed with the bridge reading at some distance from the actual e.m.f. of the cell. Coulter (6) used a quadrant electrometer to overcome this difficulty. With experience, however, the e.m.f. of a cell may be fairly accurately judged beforehand and the bridge reading adjusted to this value before closing the electrode circuit, and the real potential found. Slight polarization currents have little appreciable effect on the e.m.f. but when a larger polarization current was allowed to pass through the electrode vessel the bridge reading was readjusted and several successive

readings taken at one- or two-minute intervals until the correct e.m.f. was obtained; Eh values were calculated from the observed e.m.f.

Results

Experiments with Freshly Autoclaved Broth

In these experiments broth was used immediately after autoclaving as in the case of Coutler's experiments (5). Beef infusion broth, buffered to *p*H 7.3, was rapidly cooled after autoclaving, and electrode vessels were quickly set up and sealed with vaseline. Platinum electrodes were used in the first and mercury in the second group of experiments. In each test, at least three electrode vessels were used, one as a control, sealed with vaseline and maintained without gas, and two or more through which nitrogen was bubbled. Determinations of e.m.f. were begun at once. The results are shown in Fig. 1 and 2 where the determined Eh values are plotted as ordinates against the time as abscissae, the gas curves representing the mean of the various cells used.

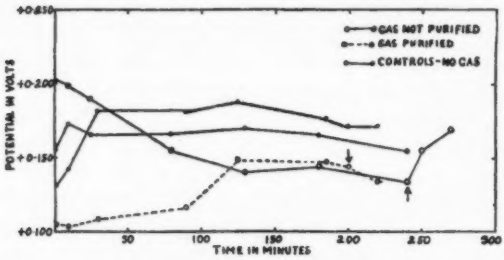

FIG. 1. *Curves showing the effect of nitrogen on a platinum electrode in buffered beef infusion broth at 0°C. The ordinates represent observed potentials in volts. The arrows indicate the point at which the gas was turned off.*

It is evident that with both metals there was considerable variation in potential in the control tubes during the first half-hour of the test. This variation apparently represents the time required for the electrode to reach equilibrium with the freshly autoclaved medium. Such potential drifts have been investigated by Dixon and Quastel (7), Kendall and Loewen (13), Conant and Fieser (4) and others. After this preliminary period the potential remained approximately constant at about +0.175 volts for platinum and +0.04 volts for mercury for the time of the experiment, four to five hours. As the curves suggest, there appears to be a very gradual negative potential drift; in certain experiments an equilibrium value of approximately −0.100 volts was reached in a week. This corresponds with the findings of Dubos (8) and Coulter (5, 6).

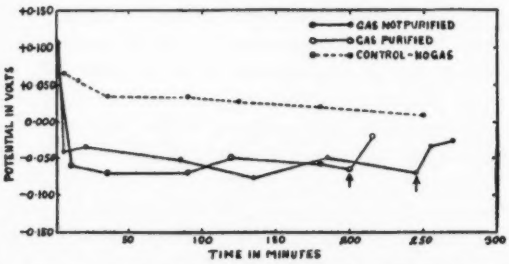

FIG. 2. *Curves showing the effect of nitrogen on a mercury electrode in buffered beef infusion broth at 0°C. The ordinates represent observed potentials in volts. The arrows indicate the point at which the gas was turned off.*

In contrast it may be observed from Fig. 1 and 2 that nitrogen caused a slight negative drift to an equilibrium potential of −0.14 volts with platinum and −0.60 volts with mercury in the experimental period of four to five hours. As in practically all cases the potential rose when the gas was turned off and as the medium without gas is known to be undergoing a gradual negative drift it is doubtful if any effect due to nitrogen other than stirring can be conceded. Experiments reported below bear out this contention. Similar results were obtained with beef extract broth.

Effect of Purification of Nitrogen

Coulter (5) used every precaution to eliminate oxygen from the nitrogen he used to "deaërate" broth in his time-potential measurements. Hence it was considered advisable to compare the effects of purified and commercial nitrogen. In the first instance commercial tank nitrogen was used; in the second series the gas was washed successively through alkaline pyrogallol, potassium permanganate and through long tubes of heated copper gauze.

No significant difference could be observed when using purified nitrogen in place of commercial nitrogen (Fig. 1 and 2). A trace of oxygen was evidently present in the tank nitrogen since the pyrogallol solution eventually became colored.

Experiments with Sterile Broth Aged Anaërobically

As stated above it was found by experiment that two procedures resulted in a greater degree of constancy in the Eh of the broth; (1) aging of the broth under anaërobic conditions (vaseline seal), and (2) holding the assembled cell under vaseline at a temperature of approximately 0° C. during the course of the experiment. In the following experiments, beef extract broth was autoclaved, covered with vaseline seal and allowed to stand a week or longer. Openings were then made through the vaseline, electrodes and bridges inserted through these and the whole quickly sealed; potential readings were continued for an hour or two or until the e.m.f. did not alter more than one or two millivolts on several successive readings at 10-min. intervals. The potential then attained in the control cell and the gas cells was recorded as the initial potential of the medium. Gas was then admitted to the gas cells as in previous experiments, and the controls maintained for the same period without changing conditions. Representative readings have been recorded in Tables I to XII.

From the results of the control determinations, without gas, it may be seen that there was remarkably slight variation in potential during the course of any one experiment with either gold or platinum electrodes and any one sample of broth. The potentials of two or more samples of broth under apparently the same conditions, however, varied considerably. This is the common observation of other investigators with such media. The causes of the variation are apparently differences in the composition and age of the broth, autoclaving, accidental admissions of air during manipulations or through an imperfect seal; and it must be recognized that vaseline dissolves oxygen and hence is rather a mechanical hindrance to diffusion of air than an air seal.

A statistical analysis of the values given in Tables I to XII and other similar data not included in the tables, gives a more concise indication of the range in potential of the broth under these conditions and the effects produced by the various gases studied. The formulae of Dunn (9) were used. The initial mean value of the potential of platinum electrodes in 14 samples of broth, calculated from the data of Tables I to XII, is 86 millivolts with a standard deviation of 77 millivolts, a probable error in the mean of 15 millivolts and a probable error in the standard deviation of 10 millivolts. Similarly the eight gold-broth potentials from the data in the same tables give a mean value of 122 millivolts with a standard deviation of 41 millivolts, a probable error in the mean of 11 millivolts and a probable error in the standard deviation of 8 millivolts. As the mercury electrode does not attain equilibrium, its determined potentials are not subject to such treatment. In other words, under similar circumstances broth may be expected to give with platinum electrodes a mean potential of 71 to 101 millivolts with a possible variation of from −100 to 163 millivolts and gold a mean potential ranging from 111 to 133 millivolts with a possible variation of from 73 to 171 millivolts.

In contrast to this unsatisfactorily large variation in the potentials of the broth from sample to sample, the variation with any one sample under the conditions specified is very much less. Thus 75 readings on three samples of broth with platinum electrodes gave, for each sample, a standard deviation of the initial mean potential of 7.2 millivolts with a probable error in the standard deviation of 1.7 millivolts. In the case of gold, 101 readings on four samples of broth showed, for each sample, a standard deviation in the initial mean potential of 22 millivolts and a probable error in the standard deviation of 4.4 millivolts. Gold electrodes appear to be much more susceptible than platinum to accidental manipulation interferences, such as moving the electrode vessel.

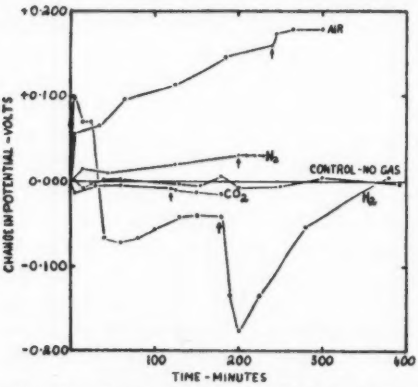

FIG. 3. *Curves showing the effect of various gases on platinum electrodes in anaerobic buffered beef extract broth at 0°C. Mean initial potentials are represented as 0 and the ordinates the changes of potentials in volts. The arrows indicate the point at which the gas was turned off.*

The introduction of air, hydrogen, nitrogen or carbon dioxide produced changes in potential at platinum, gold and mercury electrodes. The effects of these gases on the electrode potential are shown in Tables I to XII. In order that the particular effect of one gas may be more readily compared with another the initial mean potential is taken as zero and the departures from the initial mean effected by the gases calculated from the observed values shown in Tables I to XII. These data are plotted in Fig. 3, 4 and 5.

Air, as indicated in Tables I to III, and Fig. 3, 4 and 5 caused a distinct positive variation at all three electrodes, the potential tending to rise as the gas was turned off. This latter effect is probably due to the medium becoming quiescent as it has often been observed that stirring causes a negative trend in the electrode potential. It is rather remarkable, however, that the maximum positive change with air should be produced so slowly; this is a further indication of the inert nature of air on electrode potential as pointed out by French and Kahlenberg and others.

Hydrogen, as indicated in Tables IV to VI and plotted in Fig. 3, 4 and 5 produced negative drifts with gold, mercury and platinum electrodes, the effect being most pronounced with the latter metal. It is apparent that the results here reported show no effect due to hydrogen at gold or mercury electrodes beyond the standard deviation of the initial broth potentials or what may be accounted for by stirring the medium. At platinum electrodes, however, hydrogen causes a distinct and striking drop in potential comparable to the potential curve obtained with a plat-

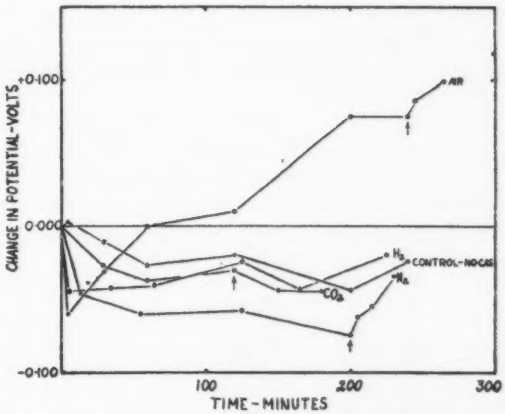

FIG. 4. *Curves showing the effect of various gases at gold electrodes in anaerobic buffered beef extract broth at 0°C. Mean initial potentials of the five lots are represented as 0 and the ordinates the changes of potential in volts. The arrows indicate the point at which the gas was turned off.*

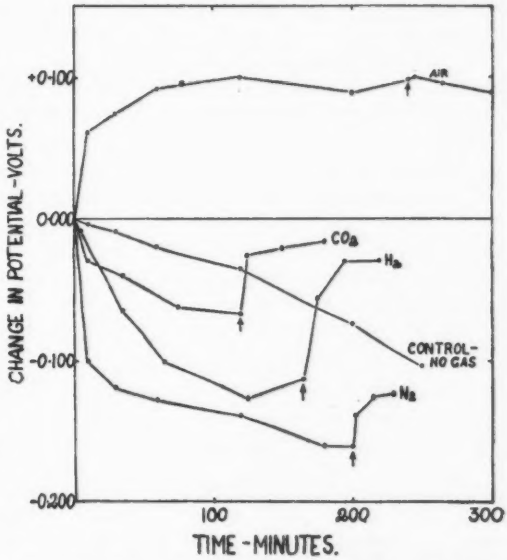

FIG. 5. *Curves showing the effect of various gases at mercury electrodes in anaerobic buffered beef extract broth at 0°C. Mean initial potentials are represented as 0 and the ordinates the change of potential in volts. The arrows indicate the point at which the gas was turned off.*

TABLE I

RESULTS OBTAINED WITH MERCURY-AIR ELECTRODE IN BUFFERED AND ANAËROBICALLY-AGED BEEF EXTRACT BROTH AT 0° C.

Time in min.	Control without gas	Sample No. 1 with air	Sample No. 2 with air	Sample No. 3 with air
	Eh in volts			
Gas on				
0	−0.049	−0.019	0.010	0.034
10	−0.053	0.010	0.130	0.067
30	−0.058	0.010	0.125	0.112
60	−0.065	0.058	0.121	0.127
120	−0.078	0.066	0.140	0.114
200	−0.087	0.021	0.151	0.115
240	−0.091	0.021	0.164	0.130
Gas off				
5	−0.080	0.030	0.147	0.147
20	−0.083	0.025	0.132	0.139
60	−0.082	0.025	0.126	0.140

TABLE II

RESULTS OBTAINED WITH GOLD-AIR ELECTRODE IN BUFFERED AND ANAËROBICALLY-AGED BEEF EXTRACT BROTH AT 0° C.

Time in min.	Control, without gas	With air
	Eh in volts	
Gas on		
0	0.182	0.141
5	0.167	0.082
30	0.160	0.110
60	0.090	0.141
120	0.070	0.152
200	0.081	0.215
240	0.086	0.217
Gas off		
5	0.096	0.228
25	0.092	0.240
60	0.096	0.260

inum electrode in hydrogen-producing cultures such as *B. coli* under anaërobic conditions. The change in potential was as great as 250 millivolts in some cases, as indicated in Table III, which is far beyond the range of standard

deviation of the medium, 7.2 millivolts. On closing off the current of hydrogen the potential falls rather than rises as in the case of the other gases. This appears to indicate definitely that the hydrogen effect is not due to stirring.

TABLE III

RESULTS OBTAINED WITH PLATINUM-AIR ELECTRODE IN BUFFERED AND ANAËROBICALLY-AGED BEEF EXTRACT BROTH AT 0°C.

Time in min.	Control, without gas	Sample No. 1 with air	Sample No. 2 with air	Sample No. 3 with air
	Eh in volts			
	Gas on			
0	0.110	0.000	0.008	0.008
5	0.111	0.087	0.090	0.009
35	0.112	0.134	0.067	0.011
65	0.109	0.190	0.090	0.024
125	0.097	0.215	0.107	0.032
185	0.105	0.226	0.204	0.027
240	0.108	0.227	0.226	0.040
	Gas off			
5	0.107	0.233	0.234	0.041
25	0.106	0.247	0.250	0.053
60	0.108	0.250	0.252	0.045

TABLE IV

RESULTS OBTAINED WITH MERCURY-HYDROGEN ELECTRODE IN BUFFERED AND ANAËROBICALLY-AGED BEEF EXTRACT BROTH AT 0° C.

Time in min.	Control, without gas	Sample No. 1 with hydrogen	Sample No. 2 with hydrogen	Sample No. 3 with hydrogen
	Eh in volts			
0	0.179	−0.014	0.043	0.080
5	0.158	0.060	0.045	0.067
35	0.152	−0.067	−0.030	0.013
65	0.137	−0.135	−0.046	−0.013
125	0.090	−0.179	−0.060	−0.035
165	−0.002	−0.148	−0.056	−0.031
	Gas off			
10	−0.012	−0.077	−0.013	0.027
30	−0.025	−0.055	0.020	0.052
55	−0.041	−0.060	0.020	0.060

TABLE V

RESULTS OBTAINED WITH GOLD-HYDROGEN ELECTRODE IN BUFFERED AND ANAËROBICALLY-AGED BEEF EXTRACT BROTH AT 0° C.

Time in min.	Control, without gas	With hydrogen
	Eh in volts	
0	0.167	0.140
5	0.170	0.100
35	0.147	0.100
65	0.147	0.100
125	0.187	0.116
165	0.170	0.097
225	0.161	0.120

TABLE VI

RESULTS OBTAINED WITH PLATINUM-HYDROGEN ELECTRODE IN BUFFERED AND ANAËROBICALLY-AGED BEEF EXTRACT BROTH AT 0° C.

Time in min.	Control, without gas	Sample No. 1 with hydrogen	Sample No. 2 with hydrogen	Sample No. 3 with hydrogen
	Eh in volts			
	Gas on			
0	0.110	0.027	0.000	0.005
5	0.111	0.110	0.090	0.130
15	0.097	0.087	0.065	0.098
25	0.106	0.085	0.053	0.084
40	0.112	−0.208	0.042	0.007
60	0.109	−0.121	0.017	−0.083
80	0.097	−0.110	0.037	−0.092
100	0.096	−0.081	0.047	−0.097
130	0.097	−0.038	0.030	−0.097
150	0.097	−0.020	0.030	−0.100
180	0.105	−0.019	0.040	−0.094
	Gas off			
10	0.107	−0.153	−0.146	−0.080
20	0.106	−0.115	−0.289	−0.080
45	0.106	−0.015	−0.288	−0.082
100	0.110	−0.023	−0.247	0.147
200	0.127	−0.025	−0.125	0.200

Fildes (10), in his work on the limiting potential required for the germination of tetanus spores used hydrogen to agitate the medium. By this procedure he produced a potential of just above zero as measured by the reduction of thionin and indophenol-1. It would seem that these dyes, which are oxidation-reduction potential indicators are not subject to the same gas effect as the

platinum electrode. Coulter (6), in fact, has shown that dye indicators and gold electrodes are not in complete agreement as regards the potential of sterile broth and suggests that dyes catalyse reactions, resulting in more negative potentials.

The other gas commonly produced by bacteria, carbon-dioxide, as shown in Tables VII to IX, has little or no effect on potential other than can be accounted for by the standard deviation of the broth electrode potentials and by the effects of stirring. Similarly nitrogen, as shown in Tables X to XII,

TABLE VII

RESULTS OBTAINED WITH MERCURY-CARBON DIOXIDE ELECTRODE IN BUFFERED AND AN AËROBICALLY-AGED BEEF EXTRACT BROTH AT 0° C.

Time in min.	Control, without gas	Sample No. 1 with carbon dioxide	Sample No. 2 with carbon dioxide	Sample No. 3 with carbon dioxide
	Eh in volts			
Gas on				
0	0.030	0.080	0.002	−0.042
10	0.038	0.017	−0.011	−0.058
35	0.020	−0.026	−0.014	−0.048
75	0.008	−0.077	−0.023	−0.050
120	0.002	−0.056	−0.030	−0.050
Gas off				
5	0.002	0.030	−0.027	−0.045
30	−0.008	0.032	−0.022	−0.038
60	−0.008	0.040	−0.022	−0.034

TABLE VIII

RESULTS OBTAINED WITH GOLD-CARBON DIOXIDE ELECTRODE IN BUFFERED AND ANAËROBICALLY-AGED BEEF EXTRACT BROTH AT 0° C.

Time in min.	Control, without gas	With carbon dioxide
	Eh in volts	
Gas on		
0	0.068	0.068
5	0.066	0.069
30	0.050	0.040
60	0.053	0.030
120	0.057	0.035
Gas off		
5	0.056	0.024
30	0.058	0.024
60	0.057	0.023

TABLE IX

RESULTS OBTAINED WITH PLATINUM-CARBON DIOXIDE ELECTRODE IN BUFFERED AND ANAËROBICALLY-AGED BEEF EXTRACT BROTH AT 0° C.

Time in min.	Control, without gas	Sample No. 1 with carbon dioxide	Sample No. 2 with carbon dioxide	Sample No. 3 with carbon dioxide
	Eh in volts			
Gas on				
0	0.117	0.177	0.226	0.110
5	0.119	0.177	0.207	0.087
30	0.115	0.178	0.241	0.083
60	0.129	0.169	0.240	0.087
120	0.135	0.165	0.243	0.076
Gas off				
5	0.131	0.165	0.244	0.070
30	0.140	0.147	0.246	0.077
60	0.140	0.123	0.246	0.084

has no effect on platinum and only a very slight negative action on gold and mercury. Coulter (5) found, when he passed nitrogen through freshly autoclaved broth, that there occurred a steady negative drop to about − 0.085 volts at a gold electrode; this he interpreted as a deaëration of the broth. This result, however, does not represent a discrepancy from the present observations since anaërobically-aged broth at a temperature of 0° C. was used. Broth, without nitrogen or, indeed, any outside influence will, under anaërobic con-

TABLE X

RESULTS OBTAINED WITH MERCURY-NITROGEN ELECTRODE IN BUFFERED AND ANAËROBICALLY-AGED BEEF EXTRACT BROTH AT 0° C.

Time in min.	Control, without gas	Sample No. 1 with nitrogen	Sample No. 2 with nitrogen	Sample No. 3 with nitrogen
	Eh in volts			
Gas on				
0	0.068	0.039	0.086	0.117
10	0.072	−0.056	0.006	−0.006
30	0.080	−0.066	−0.033	−0.018
60	0.075	−0.090	−0.030	−0.020
120	0.072	−0.075	−0.041	−0.043
180	0.032	−0.090	−0.070	−0.080
200	0.031	−0.093	−0.072	−0.074
Gas off				
2	0.031	−0.070	−0.055	−0.043
15	0.038	−0.056	−0.044	−0.036
30	0.025	−0.052	−0.045	−0.033

TABLE XI

RESULTS OBTAINED WITH GOLD-NITROGEN ELECTRODE IN BUFFERED AND ANAËROBICALLY-AGED BEEF EXTRACT BROTH AT 0° C.

Time in min.	Control, without gas	With nitrogen
	Eh in volts	
	Gas on	
0	0.084	0.124
15	0.100	0.077
55	0.102	0.065
125	0.107	0.067
200	0.117	0.054
	Gas off	
5	0.117	0.062
15	0.116	0.072
30	0.117	0.090

TABLE XII

RESULTS OBTAINED WITH PLATINUM-NITROGEN ELECTRODE IN BUFFERED AND ANAËROBICALLY-AGED BEEF EXTRACT BROTH AT 0° C.

Time in min.	Control, without gas	Sample No. 1 with nitrogen	Sample No. 2 with nitrogen
	Eh in volts		
	Gas on		
0	0.196	0.070	0.148
15	0.190	0.101	0.147
45	0.196	0.090	0.148
125	0.186	0.117	0.147
200	0.181	0.131	0.149
	Gas off		
10	0.177	0.131	0.149
20	0.179	0.132	0.148
30	0.178	0.132	0.149

ditions, attain negative potentials of the order of −0.100 volts as shown in the first section of this paper. This is in agreement with the results of Dubos (8). The anaërobic aging appears to have had in this procedure a similar effect to the passage of nitrogen in Coulter's experiments.

To summarize the results discussed in this section and shown in Fig. 3 to 5: air causes a gradual increase in positive potential with all three metals; nitrogen

and carbon dioxide show very little potential changes. This suggests that the fall in potential accompanying growth can not be accounted for by the metabolic production of carbon dioxide. Hydrogen with both gold and mercury produces little or no change in potential while with platinum it produces a conspicuous drop to a definite negative potential.

Experiments with Broth Saturated with Air

In French and Kahlenberg's work with electrodes immersed in potassium chloride solutions the electrolyte was apparently saturated with air. In order, therefore, to have a more precise comparison, anaërobically-aged broth was aërated by passing through it a gentle stream of air for four or five hours. Potential readings indicated a rise in potential of some 200 millivolts. Hydrogen was then passed into the medium and the effect noted at gold, mercury and platinum electrodes. The results are shown in Table XIII and Fig. 6. The drop in potential, *e.g.* 500 millivolts with platinum, was practically identical with that observed by French and Kahlenberg with a similar electrode in potassium chloride solution. The form of the curve varies somewhat but this might readily be expected with such widely different media as broth and potassium chloride solutions. The mercury-hydrogen electrode showed a much greater drop in potential (Fig. 6), than found by French and Kahlenberg but we have noted above that mercury apparently reacts with the broth. However, the degree of agreement in two widely different media seems to increase the significance of gas-metal electrode potentials.

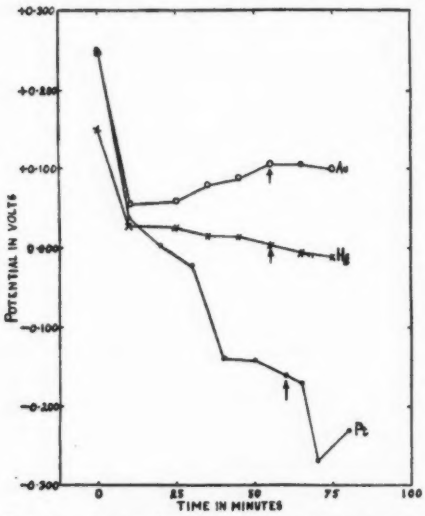

FIG. 6. *Curves showing the effect of hydrogen on gold, mercury and platinum electrodes in buffered beef extract broth previously subjected to a stream of air for 5 hr. at 0°C. The ordinates represent the potential in volts. The arrows indicate the point at which the gas was turned off.*

TABLE XIII

EFFECT OF HYDROGEN ON AIR-SATURATED BUFFERED AND PREVIOUSLY ANAËROBICALLY-AGED BEEF EXTRACT BROTH AT 0° C.

Time in min.	Mercury, Sample No.1	Mercury, Sample No.2	Mercury, Sample No.3	Platinum, Sample No.1	Platinum, Sample No.2	Gold
	Eh in volts					
	Gas on					
0	0.136	0.025	0.140	0.250	0.252	0.250
10	0.107	−0.019	0.030	0.040	0.037	0.050
35	0.090	−0.053	0.012	−0.270	−0.006	0.080
55	0.062	−0.040	−0.010	−0.260	−0.060	0.107
	Gas off					
10	0.050	−0.046	−0.010	−0.278	−0.255	0.105
20	0.031	−0.048	−0.020	−0.248	−0.208	0.100

Summary

1. A method has been described for the preparation and maintenance of buffered broth at a potential which varies from the mean value by a standard deviation of 7.2 millivolts with platinum and 22 millivolts with gold. Different samples of broth have been shown to exhibit a much greater variation in potential.

2. The effect of air, hydrogen, carbon dioxide and nitrogen on the potential difference at gold, platinum and mercury electrodes immersed in this medium has been described. Air has been shown to cause a gradual rise in potential of 100-200 millivolts at all electrodes; hydrogen to produce a fall in potential of some 250 millivolts at a platinum electrode with little effect at mercury and gold electrodes. Carbon dioxide and nitrogen have been shown to have slight effects and for the most part within the standard deviation of the broth.

3. The gas-metal electrode potentials observed in broth have been shown to be in agreement with those previously reported for electrodes immersed in potassium chloride solutions.

4. The mercury electrode was proved unreliable as the metal apparently reacts with the medium.

Acknowledgment

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THE NATURAL MICROFLORA OF THE SOIL IN RELATION TO THE FOOT-ROT PROBLEM OF WHEAT[1]

By A. W. Henry[2]

Abstract

In these studies the natural microflora of the black loam soil typical of the Edmonton district of Alberta had a marked inhibitive action on the development of the wheat foot-rotting fungus *Helminthosporium sativum* when the latter was grown directly in this soil. The severity of foot-rot infection of wheat seedlings caused by this pathogene was correspondingly reduced as a result of this action. A trace of unsterilized soil serving as a source of the saprophytic soil organisms had almost as great an influence as a relatively large amount. A similar effect on *Fusarium graminearum*, another fungous pathogene which causes foot-rot of wheat, is indicated.

Bacteria, actinomycetes and fungi isolated from black soil each had a suppressive action on *H. sativum* in the soil and reduced the severity of foot-rot infection caused by it, but the fungi were considerably more effective than the bacteria and actinomycetes tested. A combination of all of these organisms produced the most marked effect and one equivalent to that produced by the organisms of unsterilized soil.

The significance of the results in connection with the foot-rot problem of wheat is briefly discussed and their possible bearing on other plant diseases caused by soil-borne pathogenes is mentioned.

Introduction

In the fall of 1927 an investigation was begun of the effect of various soil environmental factors on the development of certain fungous pathogenes which cause foot- and root-rots of the wheat plant. Among the projects outlined one was designed to determine the possible importance of saprophytic micro-organisms common to normal soils as competitors with such pathogenes upon their introduction into the soil. The present paper constitutes a preliminary report on this project. In speaking of the natural soil flora we refer to that which exists ordinarily in the soil without the addition necessarily of any substances which might enhance its development.

The pathogenes under investigation include *Helminthosporium sativum* P.K.B., *Fusarium graminearum* Schwabe, *Ophiobolus graminis* Sacc., *Leptosphaeria herpotrichoides* de Not., and *Wojnowicia graminis* (McAlp.), Sacc. Only the first two are considered in this paper, most attention being devoted to *Helminthosporium sativum*. All of these are capable of living as saprophytes for extended periods and on various substrata. Some of them have been isolated from the soil but relatively little is known regarding their development in it. Under field conditions they must necessarily come into competition with a great variety of soil organisms once they are introduced into the soil.

There have been numerous studies made on the associations of micro-organisms, in which it has been noted that the development of one organism may be markedly affected by the presence of one or more others. Very often an inhibitory effect is exerted by one organism on another. Many studies on this

[1] *Contribution from the Department of Field Crops, University of Alberta, Edmonton, Alberta, Canada, with financial assistance from the National Research Council of Canada.*

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subject have been conducted on artificial media, though in a few cases natural substrata have been used. Numerous references on the subject are reviewed by Porter (5), Machacek (3) and Vasudeva (8). We are concerned in our discussion only with the possible inhibitive action of micro-organisms on plant pathogenes, and are particularly concerned with but one substratum, namely, the soil. Each of the authors referred to above deals with plant pathogenes, the former incidentally and the two latter in particular, especially those affecting fruits and vegetables. Porter's work is of interest here inasmuch as one of his experiments indicated that a bacterium No. 45 used, inhibited the spread of *Fusarium lini*, the flax wilt organism, in the soil, and also measurably protected flax seedlings from infection in it. The source of the bacterium is not given, though the author notes that it grows slowly in non-enriched soil and doubts if it would be useful in checking the normal soil (pathogenic?) flora. Experiments in rag-doll germinators using this bacterium also gave measurable protection of wheat seedlings from infection by *Helminthosporium*. Investigations on the factors responsible for the reduction in severity of potato scab, sometimes resulting from green manuring, are of interest. Sanford (7) obtained results which indicated that control was not effected as a result of increased soil acidity, and suggested that the antibiotic activities of certain soil micro-organisms might be responsible. He observed that a number of bacteria and fungi inhibited *Actinomyces scabies* on artificial media. Millard and Taylor (4) supplied experimental proof that a saprophytic species *Actinomyces praecox* exerted an inhibitory action on *Actinomyces scabies* and on scab formation, in soil mixed with grass and in soil without grass. It is suggested that the action on *Actinomyces scabies* results from starvation due to the exhaustion of the available food supply of the soil by the saprophytic species, and that bacteria as well as *Actinomyces* may play a part in this. Rosen and Shaw (6) studied the interaction of two soil inhabiting plant pathogenes, *Sclerotium rolfsii* and *Fusarium vasinfectum*, on artificial media, and Bamberg (1) in a study on corn smut isolated a bacterium from corn which prevented normal infection of corn plants by *Ustilago zeae* and also destroyed colonies of this fungus in culture.

Experimental Methods

An effort has been made in these studies to approximate natural conditions as closely as possible. The procedure has been to transfer the pathogenes and saprophytic organisms under test directly from agar slants to flasks of moist soil, and to allow them to develop on this substratum alone without the addition of any extraneous materials. Edmonton black loam soil has been used throughout. Small Erlenmeyer flasks each containing 50 gm. of soil and 30 cc. distilled water were used for culturing the organisms. In some cases the flasks of soil were sterilized before adding the organisms and in other cases they were not. Those which were sterilized were autoclaved for 2 hr. at 15 lb. pressure. After the organisms were added the flasks were allowed to stand at room temperature for 24 days. The contents of each flask was then deposited at seed level in a single 5-in. pot containing approximately 750 gm. of soil which

had been sterilized for 4 hr. at 15 lb. pressure. Marquis wheat seeds, previously sterilized by the modified hot water method, were then sown directly in this layer of applied soil and covered with sterilized soil. All series were started at the same time, so that all of the data given are comparable.

Saprophytic members of the natural soil flora used belong to the three general groups of organisms, namely, bacteria, actinomycetes and fungi. Representatives of these groups were isolated from samples of Edmonton black soil, which were collected in such a way as to insure that the organisms came only from the soil. The organisms were isolated in the usual manner using media specially designed for culturing the groups of organisms indicated (2). Cultures of these were grown separately on potato dextrose agar. The bacteria were added to the soil as suspensions in sterile distilled water, and the other two groups in the form of bits of the colonies. As no attempt was made, in the experiments reported, to study the effects of individual organisms only mixtures of each group and of all groups were brought together. In addition to the separate groups of organisms isolated from soils, the effects of all organisms in it were studied by using unsterilized Edmonton black soil as a source of these. In the latter case, organisms other than those referable to the plant kingdom may of course be involved.

Another point which should be borne in mind is that the pathogenes and the saprophytic organisms in these studies were added to the soil at the same time, so that the former really have more of an advantage in the competition than they would have in nature. The only exceptions are those in which the pathogenes were added to flasks of unsterilized soil.

The data are not compared statistically in this preliminary paper as the important differences were sufficiently marked and consistent to assure their significance.

The Natural Microflora of Unsterilized Soil

In order to test the hypothesis that the natural soil flora is important in retarding or preventing the development of foot-rotting pathogenes when they enter it, thus reducing the severity of their attack on wheat plants, varying amounts of unsterilized soil, together with the pathogene *H. sativum*, were added to flasks of sterilized soil. The amounts of unsterilized soil added per flask of 50 gm. of sterilized soil are indicated in Table I. The smallest portion added was that tiny amount which adhered to the tip of a moist sterilized platinum needle. On the other extreme the entire 50 gm. of soil was unsterilized. These flasks were kept at room temperature for 24 days, when attempts were made to isolate the pathogene from them. The contents of each flask was added to a pot of sterilized soil. Twenty-five sterilized Marquis wheat seeds were then sown in each pot. Each series consisted of ten pots. Notes on the seedlings were taken two weeks after planting.

If our hypothesis is well founded it would seem that there should be nearly as great a retardation of the pathogene, and consequently of disease development, in the cases where only a small amount of unsterilized soil is added as where large amounts are added, for during 24 days any organisms present in

the small amounts would have an ample opportunity of increasing and dominating the pathogene. As shown by Table I, this appears to be approximately what happened in the experiment there summarized.

TABLE I

EFFECT OF THE ADDITION OF VARYING QUANTITIES OF UNSTERILIZED SOIL TO STERILIZED SOIL ON THE DEVELOPMENT OF *H. sativum* AND OF FOOT-ROT OF WHEAT SEEDLINGS CAUSED BY THIS PATHOGENE

Series No.	Amount of unsterilized soil added per flask [1] in gm.	Recovery of pathogene from soil. Per cent successful isolations [2]	Seedling notes		
			Per cent emergence	Height in cm.	Degree of infection in %
1	none	100	74.4	11.5	47.6
2	trace	30	94.8	17.9	7.8
3	1	0	92.4	18.2	7.0
4	5	0	86.4	17.5	3.1
5	50	0	88.4	16.4	5.5

[1] *In series 1 to 4, each flask contained 50 gm. of sterilized soil at the start, while in series 5 no sterilized soil was present.*

[2] *Out of 10 trials each from a separate flask.*

A very marked difference in the severity of infection of the wheat seedlings will be noted, even with the addition of but a trace of unsterilized soil. This has resulted in an increased height and a better stand of the plants. The effect is not much more pronounced in the series which received the larger amounts of unsterilized soil. This is illustrated in Plate I, Fig. 1 and Plate II-1. In Plate II-1, all five series of the experiment are illustrated, four typical pots of seedlings from each series being shown. In Plate I, Fig. 1, typical seedlings from series 5, 2 and 1 are shown. It will be noted that in series 5 and 2 the seedlings are quite clean and practically normal whereas in series 1 they are badly stunted and rotted at the base.

The fact that the pathogene was not recovered from the soils receiving the larger amounts of unsterilized soil does not necessarily prove that it is not present in them, for the cultures were naturally quickly overgrown with saprophytic organisms. This growth may have prevented sporulation of the pathogene, thus accounting for our failure to detect it. It hardly seems possible, however, that such differences as for instance those shown between series 1 and 2 of this experiment, could be produced by any constituents of unsterilized soils other than living organisms.

The probability that similar results may be expected with pathogenes other than *H. sativum* is indicated by Table II and Plate II-3, and Plate II-4, which show the effects on wheat seedlings of culturing *Fusarium graminearum* on sterilized and unsterilized soils. Control series on sterilized and unsterilized soil, which received no pathogene, are included for comparison. These may also be compared with data in Tables I and III.

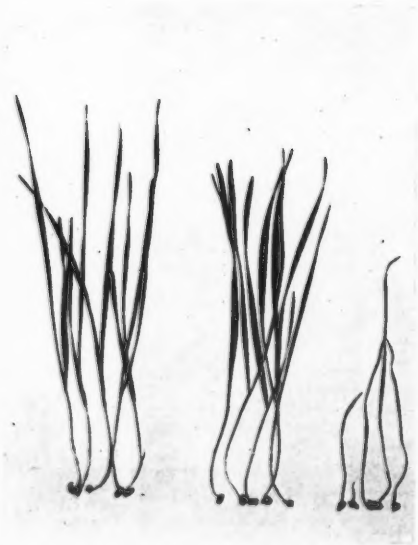

FIG. 1. *Effect of unsterilized soil in reducing the severity of foot-rot infection of wheat seedlings caused by H. sativum. Left, unsterilized soil used for culturing the pathogene. Centre, sterilized soil plus a trace of unsterilized soil used for culturing the pathogene. Right, sterilized soil alone used for culturing the pathogene.*

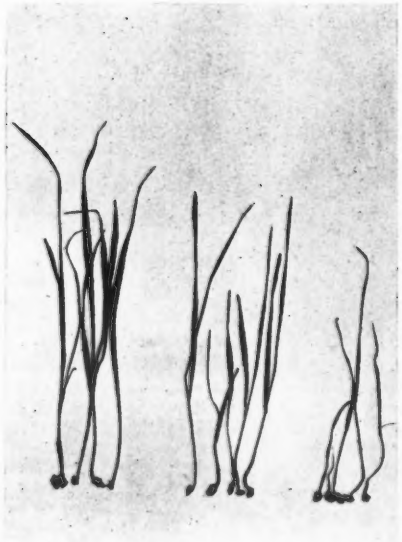

FIG. 2. *Effect of the association of H. sativum with actinomyceles and bacteria in the soil, on the severity of foot-rot of wheat seedlings caused by this pathogene. Left, after association with actinomyceles. Centre, after association with bacteria. Right, after growing alone on sterilized soil.*

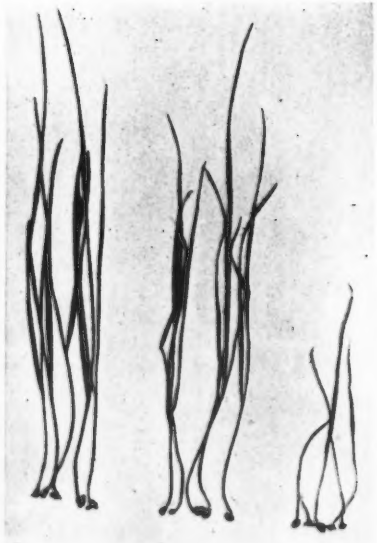

FIG. 3. *Effect of the association of H. sativum with fungi and a mixture of organisms in the soil, on the severity of foot-rot infection of wheat seedlings caused by this pathogene. Left, after association with saprophytic fungi. Centre, after association with a mixture of bacteria, actinomyceles and fungi. Right, after growing alone on sterilized soil.*

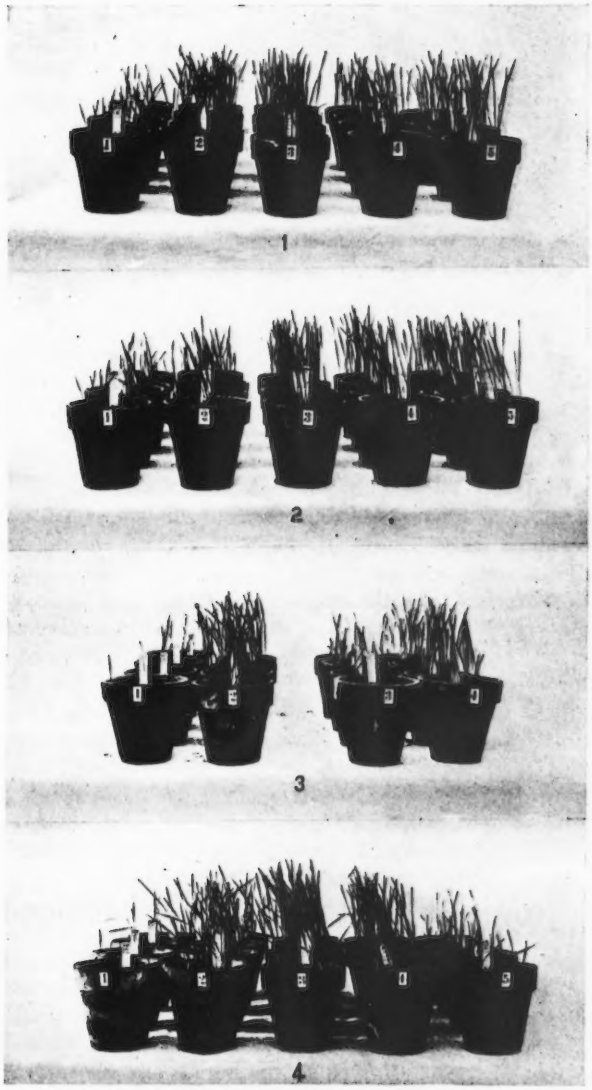

FIG. 1. *Effect of inoculating Marquis wheat with the foot-rotting pathogene Helminthosporium sativum, cultured in sterilized soil containing varying quantities of unsterilized soil. (1) Pathogene in sterile soil. (2) Pathogene in sterile soil+a trace of unsterile soil. (3) Pathogene in sterile soil+1 gm. unsterilized soil. (4) Pathogene in sterile soil+5 gm. unsterilized soil. (5) Pathogene in unsterilized soil.*

FIG. 2. *Effect of inoculating Marquis wheat with H. sativum, cultured in sterilized soil containing various saprophytic micro-organisms. (1) Pathogene in sterile soil. (2) Pathogene in sterile soil+bacteria 5 and 6. (3) Pathogene in sterile soil+actinomycetes 1 and 2. (4) Pathogene in sterile soil+fungi 3, 4, 7 and 8. (5) Pathogene in sterile soil+all bacteria, actinomycetes and fungi.*

FIG. 3. *Effect of inoculating Marquis wheat with Fusarium graminearum and Helminthosporium sativum, cultured on sterilized and unsterilized soil. (1) F. graminearum in sterile soil. (2) F. graminearum in unsterile soil. (3) H. sativum in sterile soil. (4) H. sativum in unsterile soil.*

FIG. 4. *Comparison of series of Marquis wheat seedlings inoculated with Fusarium graminearum and Helminthosporium sativum, on sterilized and unsterilized soil, with similar series lacking the pathogene and with one on sterile soil containing the pathogene plus a mixture of saprophytic soil micro-organisms. (1) F. graminearum in sterile soil. (2) No pathogene, unsterile soil. (3) No pathogene, sterile soil. (4) H. sativum+all bacteria, actinomycetes and fungi. (5) H. sativum in sterile soil.*

TABLE II

EFFECT OF THE ORGANISMS OF UNSTERILIZED SOIL ON THE DEVELOPMENT OF *Fusarium graminearum* AND OF FOOT-ROT OF WHEAT SEEDLINGS CAUSED BY THIS PATHOGENE

Treatment of soil	Recovery of pathogene from the soil. Per cent successful isolations [1]	Seedling notes		
		Per cent emergence	Height in cm.	Degree of infection in %
Sterilized	0	98.8	19.3	1.0
Sterilized + *Fusarium graminearum*	100	33.2	11.3	54.7
Unsterilized	0	89.6	16.0	5.4
Unsterilized + *Fusarium graminearum*	0	83.6	17.7	5.1

[1] *Out of 10 trials each from a separate flask.*

If these effects are due to members of the natural soil flora it would obviously be interesting to know the relative effectiveness of the different groups of organisms. The remainder of the paper deals with this question. In the experiments reported only a very few organisms in each group were tested, so that the results cannot necessarily be said to be representative of that particular group. Work with a wider range of organisms and designed to determine the effects of individual organisms will be reported later.

SOIL BACTERIA

Two isolations of soil bacteria were selected at random from plates of soil extract agar, Medium No. 7 given by Fred and Waksman (2), on which they were grown. The isolations were made on June 26, 1930, from Edmonton black soil which had been sown to wheat, and which had borne wheat the previous year. These bacteria were grown separately on potato dextrose agar until the experiments were started. They were then added to the flasks of sterilized soil in the form of suspensions in sterile water, at the same time as the pathogene *H. sativum* was added. They remained in association with the pathogene on the sterilized soil for 24 days before the contents of the flasks were transferred to pots of sterilized soil in the greenhouse, and sown to wheat as in previous experiments. The results are shown in Table III and illustrated in Plate I, Fig. 2 and in Plate II-2. It will be noted that the pathogene was reisolated successfully from every flask where it was associated with the bacteria. However, the bacteria did apparently affect the pathogene to some extent since the inoculated wheat seedlings were less severely attacked and as a result grew somewhat better than in the series where the pathogene was grown alone in sterilized soil. The seedlings were decidedly stunted, however, and some of them rotted at the base as is shown in Plate I, Fig. 2. Of all the organisms tested the bacteria appeared to have the least effect on *H. sativum* and gave, apparently as a consequence, the least protection to the wheat seedlings.

TABLE III

EFFECT OF VARIOUS MEMBERS OF THE SOIL MICROFLORA ON THE DEVELOPMENT OF *H. sativum* IN THE SOIL AND OF FOOT-ROT OF WHEAT SEEDLINGS CAUSED BY THIS PATHOGENE

Treatment of soil [1]	Recovery of pathogene from the soil. Per cent successful isolations [2]	Seedling notes		
		Per cent emergence	Height in cm.	Degree of infection in %
Control—no pathogene	0	98.8	19.3	1.0
Pathogene alone added	100	74.4	11.5	47.6
Pathogene+bacteria 5 and 6 ([3])	100	91.6	13.0	28.5
Pathogene+actinomycetes 1 and 2 ([3])	100	93.6	17.8	26.0
Pathogene+fungi, 3, 4, 7 and 8([3])	0	85.2	16.7	4.8
Pathogene+fungi, 3, 4, 7 and 8. Pathogene added 6 days after fungi	0	87.2	17.9	3.7
Pathogene+bacteria 5 and 6, actinomycetes 1 and 2 and fungi 3, 4, 7 and 8	0	95.6	18.3	2.8
Control+fungi, 3, 4, 7 and 8, no pathogene	0	87.2	16.7	2.6

[1] *Sterile soil was used in all series and H. sativum was added to all except the first one.*

[2] *Out of 10 trials each from a separate flask.*

[3] *These numbers are the culture members of the different saprophytic organisms.*

SOIL ACTINOMYCETES

The procedure with the isolations of actinomycetes was the same as with the bacteria, except that a different medium was used, namely, Medium 34 of Fred and Waksman (2). They were isolated from the same soil in a similar manner to the bacteria. The results are shown in Table III and are illustrated in Plate I, Fig. 2 and in Plate II-2 where they may be compared with those obtained with the bacteria. Attempts to reisolate the pathogene *H. sativum* after 24 days association with the actinomycetes were successful in all cases. There has, however, apparently been some effect on the pathogene as in the tests with bacteria. It has been unable to attack the wheat seedlings as severely. Measured in this way the pathogene appears to have been suppressed slightly more by the actinomycetes used, than by the bacteria.

SOIL FUNGI

Four soil fungi were isolated from the same soil as the bacteria and actinomycetes, using Medium 18 as given by Fred and Waksman (2). They belong to the three genera, *Rhizopus*, *Pencillium*, *Fusarium* and an unknown one. The latter fungus produced only chlamydospores in culture. These fungi were grown separately on potato dextrose agar and at the beginning of the experiments added in mixtures to the sterile soil at the same time as *H. sativum*. Attempts to reisolate the pathogene were unsuccessful after 24 days association with the saprophytic fungi. The agar slants were overgrown with the saprophytes as might be expected and the presence of the pathogene was not

evident. It may, however, have been present but unable to sporulate. The fact that the saprophytic fungi suppressed the pathogene very greatly is shown by the effect on the wheat seedlings, which will be noted in Table III, Plate I, Fig. 3, and Plate II-2. The degree of infection is much lower than in the series in which the pathogene was associated with either the bacteria or the actinomycetes (Fig. 4 of the text). In fact the effect on the seedlings is almost comparable to that in which the saprophytic fungi were added without the pathogene, as is shown in Table III. Of the three groups of organisms it would appear that the fungi are most active in suppressing *H. sativum*.

Mixtures of Bacteria, Actinomycetes and Fungi

In order to determine if a mixture of all of the organisms, bacteria, actinomycetes and fungi would have a different effect on *H. sativum* than any one group, all of them were added together with the pathogene *H. sativum* to a series of flasks of sterilized soil. As before, after 24 days attempts were made

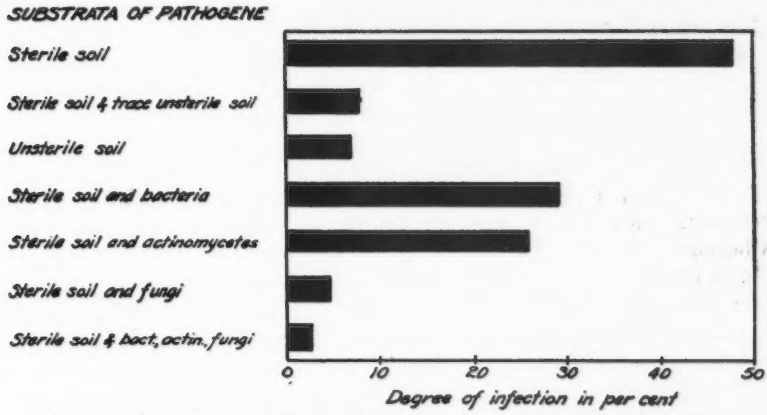

FIG. 4. *Relative degree of foot-rot infection of wheat seedlings produced, when inoculum used consisted of soil cultures of H. sativum in association with various saprophytic soil organisms.*

to isolate the pathogene from these flasks. The results were negative in all cases. Inoculations of wheat seedlings were also made as before, the results of which are shown in Table III; Plate I, Fig. 3; Fig. 4 of the text; and in Plate II-2 and Plate II-4. It is interesting to note that the seedlings in this series show the nearest approach to those of the control in freedom from disease, in per cent emergence, and in height, of any of the series to which the pathogene was added, including unsterilized soil.

Discussion

It would appear from the results of the experiments outlined above that the natural microflora of the black soil of the Edmonton district has a pronounced ability to suppress the wheat foot-rotting pathogene, *H. sativum*, in the soil.

Fusarium graminearum appears to be susceptible to a similar action. The fact that small amounts of unsterilized soil have almost as great an effect as large amounts, indicates that the saprophytic organisms in the soil are primarily concerned. Of these, it appears that all of those tested have some effect, but that the soil fungi are considerably more active in this respect than either the bacteria or the actinomyces. More organisms of each group, however, need to be tested before any general statement can be made regarding the relative inhibitive action of the different groups of organisms on plant pathogenes in the soil. It is also possible that different pathogenes may be affected differently by the same saprophyte. Additional experiments, not reported here, also show that individual members of each group of the soil microflora vary in their inhibitive action on *H. sativum*. Suppression of the pathogene in the soil appears to be correlated with a reduction in severity of its attack on wheat seedlings grown in this soil, as would be expected.

Of the various hypotheses which have been advanced by others to explain the inhibitive action of saprophytic organisms on plant pathogenes such as: a toxic action, an alteration of the pathogenicity of the pathogene and an exhaustion of the food supplies by the saprophytes, it appears probable that the latter is most important in these investigations. However, until further work is done nothing definite can be said on this question.

The fact that as great if not a greater inhibitive action on *H. sativum* was produced by a combination of the members of the various groups of the natural soil flora tested, as by the organisms of unsterilized soil, indicates that the soil flora rather than the soil fauna is primarily responsible for the effects on the pathogenes in these studies.

In conclusion the significance of the results, in connection with the foot-rot problem of wheat, may be considered briefly. In connection with experimental work, they would seem to offer at least a partial explanation of frequent failures to obtain successful infections in the field where inoculum is applied to the soil. They no doubt also explain why it is so difficult to isolate certain plant pathogenes from field soils. We have had no difficulty in isolating all of the organisms, mentioned at the beginning of this paper, from sterilized soil, but this is not true for unsterilized soil.

The results are also suggestive from the practical standpoint especially in reference to crop rotations. It is well known that several of the foot-rot diseases of wheat are much less severe when the crop is grown on summer-fallowed land, than when it is grown on land that has been cropped to wheat for several years. In the bare fallow the saprophytes of the soil no doubt have a considerable advantage over the pathogenes in the competition for food supplies. Where stubble is present on which the pathogenes are already established, the soil saprophytes probably are unable to overcome them as readily. Inasmuch as summer-fallowing is so commonly practised in western Canada, we have special reason to investigate this question further.

It is also well known that meteorological factors play an important part in the development of foot-rot diseases of wheat and other cereals. They also influence the development of the natural flora of the soil. It is quite possible

that the effects of these meteorological factors on foot-rot diseases in the field may not be entirely due to their direct action on the causal pathogenes and their host plants, but also in some cases to their indirect action on the saprophytes in the soil which in turn may affect the pathogenes and the diseases caused by them.

It is probable that the influence of the natural soil flora on soil-borne plant pathogenes other than those mentioned may be more important than is generally recognized.

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